THE TRANSGENERATIONAL EFFECTS OF DI(2-ETHYLHEXYL) PHTHALATE ON FEMALE REPRODUCTION IN MICE

BY

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DISSERTATION

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ABSTRACT

Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer used to confer flexibility and stability in common polyvinyl chloride products. DEHP can be found in numerous consumer products and is a ubiquitous chemical. Humans are exposed to DEHP daily due to its widespread use and ability to leach from out of products. DEHP and its bioactive metabolites are found in human tissues such as amniotic fluid, cord blood, breast milk, and human follicular fluid. Exposure to DEHP is a public health concern because DEHP is a known endocrine disrupting chemical and reproductive toxicant in females. The presence of DEHP in tissues vital for reproduction and development suggests that DEHP exposure occurs at various developmental stages; thus, posing as a potential toxicant for several generations.

Multiple organs are required to facilitate healthy reproduction. Organs within the hypothalamic-pituitary-ovary axis consist of the hypothalamus, anterior pituitary, and the ovary and are vulnerable to toxicant exposure. Specifically, the ovary is a heterogeneous organ composed of follicles, oocytes, corpora lutea, and interstitial tissue. The primary roles of the ovary are to produce sex steroid hormones and maintain a steady stream of growing follicles. Normal sex steroid hormone production by the mature follicles is essential for the maintenance of normal menstrual/estrous cyclicity, reproductive tract tissues, and non-reproductive tissues such as bones, the heart, and the brain. In addition, the mature antral follicle is the only follicle type capable of ovulating and releasing an oocyte for potential fertilization. Therefore, female fertility is dependent on the maintenance of a constant stream of growing follicles and sex steroid hormone secretion throughout the reproductive lifespan.

The ability of endocrine disruptors to cause disease and infertility is a major concern, but even more troubling is that some of the effects of DEHP may be multigenerational or

transgenerational in nature. To obtain a multigenerational or transgenerational effect, the phenotype or the effect must be passed from one generation to a subsequent generation. During prenatal exposure, the F0 generation is exposed to DEHP during pregnancy. The F1 generation is exposed as a developing fetus and the F2 generation is exposed as the developing germ cells inside the fetus. Both the F1 and F2 generations are directly exposed and effects observed in these generations are considered multigenerational effects. The subsequent generation, the F3 generation, is the first generation that is not directly exposed to DEHP and any effects observed are considered transgenerational effects. The transgenerational phenomena does not involve direct exposure and usually involves epigenetic changes induced in the germline.

Limited information is available on the effects of environmentally relevant DEHP exposure on female reproduction and even less information is available on the effects of DEHP on female reproduction across generations. Therefore, the goal of my doctoral dissertation work was to investigate the multigenerational and transgenerational toxicant effects of DEHP on female reproduction. Specifically, I investigated the effects of prenatal and ancestral DEHP on ovarian steroidogenesis and folliculogenesis and how DEHP exposure affects female reproductive outcomes in the F1 – F3 generation of mice. Additionally, I investigated how prenatal and ancestral DEHP exposure disrupts gene expression and DNA methylation in juvenile mouse ovaries across generations.

First, I tested the hypothesis that prenatal and ancestral exposure to environmentally relevant doses of DEHP decreases folliculogenesis and impairs steroidogenesis in the F1 – F3 generations. Folliculogenesis is the process in which follicles in the ovary develop and mature towards ovulation to release the oocyte for fertilization. Steroidogenesis involves the production of sex steroid hormones by the mature follicle type. I found that prenatal DEHP exposure

decreased folliculogenesis and the percentage of atretic follicles in adult ovaries the F1 generation. DEHP exposure disrupted folliculogenesis in adult ovaries in the F2 generation. Ancestral DEHP exposure accelerated germ cell transition into primordial follicles in neonatal ovaries in the F3 generation. I also found that prenatal DEHP exposure increases serum 17β-estradiol levels in the F1 generation and altered serum progesterone levels in the F2 generation.

Next, I tested the hypothesis that prenatal and ancestral DEHP exposure to environmentally relevant doses impair reproductive outcomes in the F1 – F3 generations of female mice. Sex organs are particularly sensitive to endocrine disruptors and during a developmental window of exposure. I found that prenatal and ancestral DEHP exposure caused precocious puberty and disrupted normal estrous cyclicity in all three generations of mice. I also observed changes in birth outcomes such as increased litter size in the F2 generation and increased percentage of female pups per litter in the F3 generation. Additionally, I found that prenatal DEHP exposure decreased fertility in the F1 and F2 generations. Specifically, prenatal DEHP exposure decreased the mating index and pregnancy rate in the F1 generation and decreased the gestational index in the F2 generation.

Further, I tested the hypothesis that prenatal and ancestral exposure to environmentally relevant doses of DEHP differentially expressed genes in pathways critical for ovarian functions and increased DNA methylation in whole ovaries in the F1 – F3 generations. I found that prenatal and ancestral DEHP exposure disrupted gene expression in various pathways in the ovary. In the F1 generation, prenatal DEHP exposure increased the expression of *Dnmt1* and increased the percentage of 5-mC in the whole ovary. In the F2 generation, DEHP exposure decreased the expression of *Tet*s. In the F3 generation, ancestral DEHP exposure decreased the expression of *Dnmt*s, *Tet*s, and decreased the percentage of 5-mC in the whole ovary.

Collectively, my doctoral dissertation work shows that prenatal and ancestral exposure to environmentally relevant doses of DEHP cause multigenerational and transgenerational impairment of ovarian health and function, female reproductive outcomes, ovarian gene expression, and ovarian DNA methylation levels.

To Moose, Jacob, and Rob.

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CHAPTER 1

Overview

1.1 Overview

Phthalates are synthetic endocrine disrupting compounds commonly used as plasticizers to confer flexibility and stability in consumer products including children's toys, food containers, furniture, personal care products, medical devices, and housing materials [1, 2]. Phthalate exposure is associated with multiple adverse health effects in humans [3-5] and experimental animals [6]. DEHP is a commonly used phthalate, di(2-ethylhexyl) phthalate (DEHP), and a known developmental and reproductive toxicant [7-9]. Specifically, in females, DEHP exposure reduces folliculogenesis and steroidogenesis *in vitro* [10], and DEHP exposure in adulthood alters estrous cyclicity, accelerates follicle recruitment [11], and accelerates reproductive aging in mice [8].

DEHP and its bioactive metabolites are found in human tissues such as amniotic fluid, cord blood, breast milk, and human follicular fluid [2]. The presence of DEHP in tissues vital for reproduction and development suggests that DEHP exposure occurs at various developmental stages; thus, posing as a potential toxicant for several generations. Numerous studies have examined the effects of prenatal exposure to DEHP and its metabolites on the offspring. In animal studies, prenatal exposure to DEHP induces premature reproductive senescence in male offspring [9] and leads to cryptorchidism in male rats [12]. Human studies show that DEHP exposure in pregnant women is associated with adverse outcomes in the offspring [13-16]. Specifically, prenatal DEHP exposure is negatively associated with birth weight [16] and head circumference [17] and with increased odds of male newborn genital anomaly [14]. These

studies demonstrate that prenatal exposure to DEHP and its metabolites negatively impact the next generation.

Despite the importance of fertility in females across generations, little is known about the mechanism by which exposure to environmental chemicals impacts female reproduction in each generation. Previous studies in male rodents suggest that epigenetic changes in DNA methylation can induce transgenerational effects. In male rats, prenatal exposure to DEHP induces epigenetic transgenerational inheritance of cryptorchidism by increasing differentially methylated DNA sequences in the testes [12]. Disruption of DNA methylation is an inheritable epigenetic modification and can be passed to future generations. In females, however, the epigenetic mechanism of action of DEHP is not known. It is critical to elucidate the epigenetic mechanisms underlying the DEHP-induced transgenerational effects in females to better understand the impacts of chemical exposure in future generations.

Therefore, the goals of these studies were to investigate effects of prenatal and ancestral exposure to DEHP on female reproductive functions and the underlying mechanisms by which DEHP exposure impacts the ovary in each generation. This was done by testing the hypothesis that prenatal and ancestral exposure to DEHP causes transgenerational effects on folliculogenesis, steroidogenesis, fertility, and the epigenome of the ovary the F1, F2, and F3 generations of female mice. To test this hypothesis, I completed the following specific aims:

Specific Aim 1: Determine if prenatal and ancestral exposure to DEHP reduces the number of healthy ovarian follicles and serum sex steroid hormone levels in the F1, F2, and F3 generations.

To complete this aim, I orally dosed pregnant CD-1 mice with vehicle control (tocopherol-stripped corn oil) or DEHP (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, or 750

mg/kg/day) daily from gestation day 10.5 until birth. Following dosing, pregnant mice gave birth to the F1 generation and females were used to produce the F2 generation. The F2 females were used to produce the F3 generation. I then examined the effects of prenatal and ancestral DEHP exposure on folliculogenesis and sex steroid hormone levels. On postnatal days 1, 8, 21, and 60, ovaries were collected and used for histological evaluation of follicle numbers and sera were used to measure progesterone, testosterone, estradiol, luteinizing hormone, and follicle stimulating hormone levels. In the F1 generation, prenatal exposure to DEHP disrupted organ weights (20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day), decreased folliculogenesis (200 µg/kg/day and 750 mg/kg/day), and increased serum estradiol levels (20 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day). In the F2 generation, exposure to DEHP decreased body weights (20 µg/kg/day) and organ weights (200 µg/kg/day), dysregulated folliculogenesis (20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day), and disrupted serum progesterone levels (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, 750 mg/kg/day). In the F3 generation, DEHP exposure accelerated folliculogenesis (500 mg/kg/day and 750 mg/kg/day). These data suggest that prenatal exposure to DEHP leads to adverse multigenerational and transgenerational effects on ovarian function. These data are presented in Chapter 3.

Specific Aim 2: Determine if prenatal exposure to DEHP leads to infertility or reproductive complications in the F1, F2, and F3 generations.

To complete this aim, I orally dosed pregnant CD-1 mice with vehicle control (tocopherol-stripped corn oil) or DEHP (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, or 750 mg/kg/day) daily from gestation day 10.5 until birth. Following dosing, pregnant mice gave birth to the F1 generation and females were used to produce the F2 generation. The F2 females were used to produce the F3 generation. In the F1 – F3 generations, the onset of puberty, estrous

cyclicity, time for mice to become pregnant, birth outcomes, and fertility related indices were collectively measured from 3 to 9 months of age. In the F1 generation, prenatal DEHP exposure accelerated the onset of puberty (200 µg/kg/day), caused abnormal estrous cyclicity (200 µg/kg/day and 500 mg/kg/day) and disrupted fertility-related indices (20 µg/kg/day and 200 µg/kg/day). In the F2 generation, DEHP exposure accelerated the onset of puberty (500 mg/kg/day), caused abnormal estrous cyclicity (20 µg/kg/day and 200 µg/kg/day), increased the litter size (20 µg/kg/day and 500 mg/kg/day), and disrupted fertility-related indices (500 mg/kg/day). In the F3 generation, DEHP exposure accelerated the onset of puberty (20 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day), caused abnormal estrous cyclicity (20 µg/kg/day), and decreased female pup anogenital index (750 mg/kg/day). Collectively, the data show that prenatal DEHP exposure at environmentally relevant doses accelerates the onset of puberty, disrupts birth outcomes, and disrupts fertility-related indices; thus, suggesting that DEHP causes female reproductive problems in a multigenerational and transgenerational manner. These data are presented in Chapter 4.

Specific Aim 3: Determine if prenatal and ancestral exposure to DEHP reduces mRNA of key ovarian regulators and induces differential methylation in the F1, F2, and F3 ovaries.

To complete this aim, I orally dosed pregnant CD-1 mice with vehicle control (tocopherol-stripped corn oil) or DEHP (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, or 750 mg/kg/day) daily from gestation day 10.5 until birth. Following dosing, pregnant mice gave birth to the F1 generation and females were used to produce the F2 generation. The F2 females were used to produce the F3 generation. At postnatal day 21 for each generation, mice were euthanized and ovaries were removed for gene expression analysis of various ovarian pathways via qPCR and 5-mC quantification. In the F1 generation, prenatal DEHP exposure disrupted the expression of

cell cycle regulators (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day), decreased the expression of peroxisome-proliferator activating receptors (20 µg/kg/day and 750 mg/kg/day), and increased the percentage of 5mC in the ovary (20 µg/kg/day) compared to control. In the F2 generation, exposure to DEHP decreased the expression of steroidogenic enzymes (20 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day), dysregulated the expression of PI3K-pathway factors (20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day), decreased the expression of apoptosis factors (20 µg/kg/day), and decreased the expression of *Tet* (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day) compared to controls. In the F3 generation, ancestral DEHP exposure decreased the expression of steroidogenic enzymes (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day), decreased the expression of PI3K-pathway factors (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day), decreased the expression of cell cycle regulators (20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day), decreased the expression of apoptosis factors (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day), decreased the expression of *Esr2* (20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day), decreased the expression of DNA methylation factors (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day), and decreased the percentage of 5mC (500 mg/kg/day and 750 mg/kg/day) compared to controls. Overall, the data show that prenatal and ancestral DEHP greatly suppresses gene expression of pathways required for folliculogenesis and steroidogenesis in the ovary in a transgenerational manner and that gene expression may be influenced by DNA methylation. These results provide insight into some of the mechanisms of DEHP-mediated toxicity in the ovary across generations. These data are presented in Chapter 5.

Collectively, this work determined that prenatal and ancestral exposure to DEHP disrupts the expression of pathways important for normal ovarian function and it causes epigenetic changes

in the ovary of subsequent generations. This study provides a better understanding of how DEHP exerts transgenerational effects on reproduction.

In summary, Chapter 1 provides an overview of my dissertation. Chapter 2 provides background information on phthalates, endocrine disruption, transgenerational epigenetic inheritance, female reproduction, and the epigenetic effects of endocrine disruption on female reproduction. Chapter 3 describes the work that tests the hypothesis that prenatal exposure to DEHP disrupts ovarian function in a transgenerational manner in female mice. Chapter 4 describes the work that tests the hypothesis that DEHP exposure during prenatal development causes adverse transgenerational effects on female fertility in mice. Chapter 5 describes the work that elucidates the mechanisms by which DEHP exposure disrupts ovarian function across three generations. Finally, Chapter 6 summarizes the findings presented in the dissertation and outlines future study directions.

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CHAPTER 2

The Epigenetic Impacts of Endocrine Disruptors on Female Reproduction Across Generations

2.1 Abstract

Humans and animals are repeatedly exposed to endocrine disruptors, many of which are ubiquitous in the environment. Endocrine disruptors interfere with hormone action, thus causing non-monotonic dose responses that are atypical of standard toxicant exposures. The female reproductive system is particularly susceptible to the effects of endocrine disruptors. Likewise, exposures to endocrine disruptors during developmental periods are particularly concerning because programming during development can be adversely impacted by hormone level changes. Subsequently, developing reproductive tissues can be predisposed to diseases in adulthood and these diseases can be passed down to future generations. The mechanisms of action by which endocrine disruptors cause disease transmission to future generations are thought to include epigenetic modifications. This review highlights the effects of endocrine disruptors on the female reproductive system, with an emphasis on the multi- and transgenerational epigenetic effects of these exposures.

2.2 Endocrine Disruptors

Synthetic chemicals have become a part of people's everyday lives and some of these chemicals have been identified as endocrine disruptors. Endocrine disruptors are exogenous chemicals, mixtures of chemicals, or non-chemical exogenous factors that interfere with the body's normal endocrine system, leading to adverse effects on hormonally controlled functions [1]. Endocrine disruptors are heterogeneous and vary from synthetic to natural chemicals. Specifically, synthetic chemicals such as polychlorinated biphenyls, plasticizers, pesticides, fungicides, and pharmaceutical agents are known endocrine disruptors [1]. Natural chemicals such as phytoestrogens found in food are also known endocrine disruptors. These chemicals serve various purposes and are ubiquitous in the environment [1]. Endocrine disruptors interfere with hormone actions by mimicking hormones, promoting inappropriate responses at improper times, or by blocking hormone action, leading to alterations in the hormonal and homeostatic systems and interfering with the ability of the body to communicate with and respond to the environment [1]. Endocrine disruptors tend to have a low binding affinity for hormone receptors and their ability to activate or block hormone receptors may vary. Although it is often difficult to define adverse effects, some researchers consider any biological response to an endocrine disruptor to be an adverse event [2].

Endocrine disruptors are prevalent in the environment. These disruptors are found in food, consumer products, water, soil, and in wildlife and people who are exposed through ingestion, inhalation, dermal contact, or injection [1]. Examples of endocrine disruptors vary from chemical to non-chemical exogenous factors [1, 3]. Chemical endocrine disruptors can be categorized into three major groups: pesticides, chemicals in consumer products, and food contact materials [4]. Examples of pesticides that induce endocrine disruptive activities include

glyphosate, dichlorodiphenyltrichloroethane, atrazine, chlorpyrifos, and methoxychlor [4-6]. Endocrine disruptors found in consumer products include, but are not limited to, brominated flame-retardants, phthalates, parabens, heavy metals, polychlorinated biphenyls, nonylphenols, diethylstilbestrol, and perfluorochemicals [4, 7-9]. Additional endocrine disruptors described as food contact materials are bisphenol A, phthalates, and phenols [4, 8]. Non-chemical endocrine disruptors include light at night, improper nutrition, stress, and diet [3, 10-13].

Numerous endocrine disruptors exist, but this review will focus on several well-documented endocrine disruptors (Table 2.1). Specifically, bisphenol A (BPA) is a well described endocrine disruptor [14]. BPA is a synthetic chemical used mostly in polycarbonate plastics, epoxy resin liners in aluminum cans, and thermal receipts. It can act through various sex steroid hormone receptors, including estrogen receptors 1 and 2, androgen receptors, and thyroid hormone receptors [15]. Phthalates, like BPA, are a class of chemicals that serve as plasticizers and act as endocrine disruptors [16, 17]. Diethylstilbestrol (DES) is another example of an endocrine disruptor [16]. It was a pharmaceutical agent used as an anti-abortive drug until the 1970s, but it is no longer used due to its reproductive toxicity [16]. Dichlorodiphenyltrichloroethane (DDT) and its metabolite dichlorodiphenyldichloroethylene (DDE) are organochlorine insecticides and are well known endocrine disruptors [16, 18, 19]. Methoxyclor is another organochlorine pesticide and endocrine disruptor that was intended to replace DDT, but is now banned in many countries due to its toxicity [19]. Vinclozolin is a dicarboximide fungicide used in agriculture, but more specifically in the viniculture industry, and it exhibits endocrine disrupting effects [20]. Further, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a potent polychlorinated dibenzodioxin and endocrine disruptor. It is formed as a by-product of organic combustion and was a major component of the Agent Orange that was used

during the Vietnam War. It also was a major environmental contaminant from an industrial explosion in Seveso, Italy [16, 21].

Endocrine disruptors cause nonmonotonic dose responses such as sigmoid, U-shaped, or inverted-U-shaped curves [1, 22]. For example, xenobiotics that mimic estrogen stimulate MCF-7 human breast cancer cell proliferation at low doses, but saturate the cell growth response and do not increase proliferation at high doses [23]. Although the mechanisms behind such nonmonotonic effects are not fully understood, they may be due to receptor type and abundance in specific cells or tissues [24], receptor down-regulation and desensitization [25, 26], and endocrine feedback loops [27, 28].

Endocrine disruptors also have been shown to act at low levels and in the range of normal human exposure [1]. This is not surprising because endocrine disruptors mimic endogenous hormones, which act at low concentrations. Therefore, studies that utilize environmentally relevant and low doses are important for understanding the effects of endocrine disruptors in the body.

Recently, several studies have linked exposure to endocrine disruptors to adverse reproductive outcomes. For example, the incidence and prevalence of diseases involving reproductive tissues such as breast cancer, prostate cancer, and polycystic ovarian syndrome have increased over time [29-31]. These diseases are influenced by both genetic and environmental factors, but dramatic increases in incidence and prevalence suggest that these diseases are largely due to environmental factors. An analysis of about 44,800 pairs of twins from Sweden, Denmark, and Finland shows that the environment, and not genetics, substantially influenced the rates of sporadic prostate and breast cancers [30]. Another study demonstrates that

exposure to bisphenol A, an endocrine disruptor, plays a major role in polycystic ovarian syndrome pathogenesis [29].

The reproductive system is especially vulnerable to endocrine disruptors during development. The developmental origins of health and disease (DOHaD) is a paradigm in which environmental exposures during development can lead to health and disease risk later in childhood and adult life [32]. The concept is that environmental stressors including malnutrition and exposure to environmental endocrine disruptors during critical periods of development cause subtle changes in gene expression that lead to permanent alterations in an organ, tissue, or structure. The alteration will then lead to a health and disease risk later in life [32-34]. In addition, DOHaD disease risk can be transmitted across generations [32].

Endocrine disruptors act through multiple pathways to influence developmental programming. For example, during early development, the fetus is protected from exogenous estrogens by a plasma protein, α-fetoprotein, which binds estrogens and protects the fetus [35]. Some endocrine disruptors bypass α-fetoprotein due to weak binding affinity, subsequently rendering the fetus vulnerable to toxicity [36]. Further, other hormone-binding proteins circulate through the blood and endocrine disruptors may bind to these proteins, disrupting the balance between hormone-binding proteins and endogenous hormones [37]. Further, endogenous hormones may be less bioavailable, whereas the endocrine disruptors are physiologically available, causing inappropriate hormone signaling [37, 38]. The ability of endocrine disruptors to interfere with hormone levels during development is of concern because cell differentiation and tissue development can be adversely impacted by hormone level changes. Subsequently, these tissues can be predisposed to diseases in adulthood and disease can be passed down to future generations [39].

2.3 Epigenetic Mechanisms

The ability of endocrine disruptors to cause disease and infertility is a major concern, but even more troubling is that some of the effects of endocrine disruptors may be multigenerational or transgenerational in nature. To obtain a multigenerational or transgenerational effect, the phenotype or the effect must be passed from one generation to a subsequent generation. Multiple exposure paradigms produce multigenerational and transgenerational effects.

The first paradigm is by adult life exposure. The F0 generation is exposed to an endocrine disruptor during adult life. During this window, the F1 generation experiences preconception exposure as the germ-line. Once the F0 generation produces the F1 generation, any effects observed in the F1 generation are due to multigenerational effects of endocrine disruptor exposure. To observe a transgenerational effect from adult exposure, the subsequent generation, the F2 generation, must be produced. This is the first generation that is not directly exposed to the endocrine disruptor and any effects observed in this generation are considered transgenerational effects [40-42].

The second exposure paradigm is by prenatal exposure (Figure 2.1). The F0 generation is exposed to an endocrine disruptor during pregnancy. During this exposure window, the F1 generation is exposed as a developing fetus and the F2 generation is exposed as the developing germ cells inside the fetus. Both the F1 and F2 generations are directly exposed and effects observed in these generations are considered multigenerational effects. The subsequent generation, the F3 generation, is the first generation that is not directly exposed to the chemical and any effects observed are considered transgenerational effects [40-42]. The transgenerational phenomena does not involve direct exposure and usually involves epigenetic changes induced in the germline [39, 43-45].

Epigenetics are mitotically and meiotically heritable changes in gene function without changing DNA sequences [46, 47]. Broadly, these heritable changes in the epigenome define and control cell and tissue development by controlling gene expression [48]. Multiple molecular mechanisms alter the epigenome, including changes DNA methylation, chromatin modification, and noncoding RNAs (Figure 2.2). Epigenetic modifications must be transmitted through the germline to the unexposed generation to cause a transgenerational phenomenon [41].

DNA methylation is a commonly studied epigenetic mechanism [49]. Specifically, methylation of DNA is a highly dynamic modification that occurs on the cytosine residue in "CpG" dinucleotides [50]. CpG indicates cytosine-phosphate-guanine and that these nucleotides are in sequence next to each other as opposed to CG base pairs across the DNA strand [49]. CpG sites are frequent in the promoter regions of some genes and are defined as CpG islands. Cytosines in 5' promotor region that are methylated hinder the transcription of the gene, thus causing gene silencing. This is because DNA methylation is associated with providing a physical barrier, which impedes transcription factor binding, resulting in a downregulation of gene expression [51, 52]. Downregulation of gene expression can be due to a steric hindrance of the transcription factor binding to the promotor region of the gene or a secondary recruitment of DNA binding proteins that prevent transcription factors from binding to the DNA [49]. However, cytosine nucleotides may be demethylated. Demethylation of cytosine nucleotides in CpG islands allows access of transcription factors to the promoter region, typically resulting in an upregulation of gene expression [51, 52].

DNA methylation is performed by a group of enzymes named DNA methyltransferases (DNMTs). The main classes of DNMTs are DNMT1 and DNMT3 [53, 54]. DNMT1 is the maintenance DNMT because it maintains the original DNA methylation pattern in a cell lineage.

It primarily serves to methylate CpG sites during DNA replication so that both daughter cells have the same DNA methylation patterns [54-56]. DNMT3a and DNMT3b are methyltransferases that methylate CpG sites on naked DNA outside of DNA replication. These DNMTs are critical for early development. Besides tissue distribution, little difference exists between DNMT3a and DNMT3b. DNMT3a is ubiquitously distributed, whereas DNMT3b is restricted to the thyroid, testes, and bone marrow [53, 57, 58]. DNMT3s methylate DNA by two interlinked mechanisms. Specifically, DNMT3a and DNMT3b may be recruited to promoters by specific transcription factors or the DNMTs may methylate all CpG sites not protected by a transcription factor in the genome [57, 59, 60].

The covalent bond between a methyl group and the 5’ position of cytosine is one of the strongest bonds in nature. It is nearly impossible to break this bond; thus, DNA methylation can be a permanent epigenetic modification passed to future generations. However, DNA methylation is not always permanent and the DNA can undergo demethylation. In fact, DNA demethylation is important in the early stages of development, in highly specialized postmitotic cells, and occurs as a response to extrinsic signals [61]. DNA demethylation can occur through passive or active action. Passive DNA demethylation occurs through an absence of active DNMT1 proteins during DNA synthesis. Without the maintenance DNMT, DNA in dividing cells will not pass the methylation pattern on to the daughter cells. This will result in a progressive loss of methylated CpGs and consequently reduce the methylation markers on cytosines following each cell division. Passive demethylation occurs in activing dividing cells, but does not occur in terminally differentiated cells or during active demethylation in the zygote [62, 63].

Active DNA demethylation may generally occur by one of two methods. The first is by chemically modifying the 5-methyl cytosine by deamination and/or oxidation and converting it into a thymine. A guanine/thymine DNA mismatch occurs and induces the base excision repair (BER) pathway to correct the base into a naked cytosine [57, 61]. The second active DNA demethylation method is mediated by the ten-eleven translocation (TET) enzymes. TET enzymes add a hydroxyl group onto the 5'-methylcytosine and convert it to 5'-hydroxymethylcytosine. The methylated cytosine is converted into a naked cytosine by one of two routes. The first method is that the 5'-hydroxymethylcytosine is further oxidized to 5'-formylcytosine and then into 5'-carboxycytosine [64].The BER pathway is activated and the 5-carboxycytosine is cleaved by thymine DNA glycosylase (TDG) and is replaced with a naked cytosine [65]. The second method of TET DNA demethylation is by deaminating 5'-hydroxymethylcytosine by activation-induced cytidine deaminase (AID) and apolipoprotein B mRNA-editing catalytic polypeptides (APOBECs) to form 5'-hydroxymethyluracil [61, 66]. The BER pathway is activated and the 5'-hydroxymethyluracil is cleaved by TDG and is replaced with a naked cytosine [67]. DNA demethylation occurs through multiple pathways, utilizing numerous enzymes and proteins and resulting in a dynamic, complex process.

In addition to epigenetic modifications occurring by DNA methylation, they can occur due to chromatin modifications. Chromatin modifications are epigenetic modifications that directly regulate the packaging of DNA. Over 3 billion base pairs are contained in chromosomes and need to fit inside the nucleus. Chromatin serves to compact the DNA while allowing transcription factor access to relevant DNA sequences. Chromatin is made of DNA, histone proteins, and nucleosomes. DNA is wound around an octamer of histone proteins. Histone octamers consist of four core histones: H4, H3, H2a, and H2b. H4 and H3 pair together and H2a

and H2b pair together. Each histone is found in duplicate. A fifth histone, H1, links the histone cores to each other. DNA wrapped around an octamer of histones is referred to as the nucleosome. The most important epigenetic role of chromatin is to regulate gene expression by controlling the access of transcription factors to DNA. Chromatin may be tightly compacted, blocking transcription factor access to DNA, and reducing gene transcription. Alternatively, chromatin may become loose, allowing transcription factor access to DNA, and allowing gene transcription [49].

Histones are critical for condensing DNA and their functions are primarily controlled by modifying the N-terminus, also known as histone tail domains. These histone tails are modifiable by nonhistone proteins and these modifications reflect DNA compaction. The type of modification on the specific histone and the position of the modification influences gene expression. Histone tail modifications include acetylation, methylation, proline isomerization, SUMOlyation, ubiquitination, phosphorylation, ADP ribosylation, and deamination [68]. Post-translational modifications interact with the histone tails to modify the transcriptional regulatory readout and may occur on any of these histones [68]. Further, the position of the post-translational modification can influence the transcriptional regulatory readout. For example, two post-translational modifications on the same histone may provide a different readout compared to the same post-transcriptional modifications on two adjacent histones [69]. The two most well-known post-transcriptional modifications are acetylation and methylation.

Histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs) are enzymes that facilitate acetylation onto the lysine residues on the histone tail. Generally, acetylation of histone tails enhances gene transcription by neutralizing the charge of the lysine residue. Further, removal of the acetyl group represses gene transcription [70]. Histone

methyltransferases (HMT) and histone demethyltransferases (HDMT) are enzymes that facilitate methylation on the lysine, histidine, and arginine residues on the histone tail. Histone methylation generally leads to repressed gene transcription. However, methylation can be both activating and repressing, depending on which residue is modified and how many methyl modifications occur [70]. For example, trimethylation of the lysine 27 residue of histone H3 silences genes, whereas methylation and trimethylation of the lysine 4 residue on histone H3 activate enhancers [71].

Another type of epigenetic modification involves noncoding RNA. Noncoding RNAs (ncRNA) are forms of epigenetic modifications that serve housekeeping and regulatory functions and are involved in chromatic function. Noncoding RNAs are a large group of RNAs that fall into two main classes based on size or function. The main classes are long noncoding RNAs (lncRNAs), which are greater than 200 nucleotides in length and short noncoding RNAs, which include microRNA (miRNA), transferRNA (tRNA), piwi-interactingRNA (piRNA), and short-interferingRNA (siRNAs) [49, 72].

Long noncoding RNAs are longer than 200 nucleotides in length, do not encode protein, and mediate gene expression. Long noncoding RNAs and mRNAs are similar to each other. Both are transcribed by RNA polymerase II from genomic loci and both are biochemically similar except that lncRNA lack codons that encode proteins [73]. However, a major difference between lncRNA and mRNA is that lncRNA serve as epigenetic modifiers. The mechanisms by which lncRNAs exert their epigenetic effects are diverse. Long noncoding RNA have the ability to interact with genomic loci and chromatin by forming stable domains for protein binding and chromatin localization. Therefore, the lncRNA interactions allow sequence specific localization of chromatin-modifying complexes and the lncRNAs direct the chromatin-modifying complexes

to target genes, thus regulating transcriptional activity [74, 75]. Further, lncRNAs can directly interact with DNMT1 and affect global methylation patterns [76]. Finally, lncRNAs have been shown to interact with other noncoding RNAs such as miRNA. Specifically, lncRNA can bind and sequester miRNA and prevent them from binding to their target mRNAs [77, 78].

MicroRNA are ~22 nucleotides in length and regulate protein-coding genes. MicroRNA are first synthesized in the nucleus and then mature within the cytoplasm. Genes encoding miRNAs are transcribed into primary miRNAs (pri-miRNA) by RNA polymerase II. Pri-miRNAs are cleaved by a nuclear microprocessor complex and exported from the nucleus into the cytoplasm as pre-miRNA. Pre-miRNAs are cleaved by the protein DICER, generating a small miRNA duplex. The miRNA duplex is loaded onto an argonaute family protein (AGO) to form an effector complex called RNA-induced silencing complex (RISC) [79]. The RISC complex regulates gene expression by hybridizing to sequence-specific target mRNA and regulates transcriptional and translational functions and mRNA stabilization [80]. MicroRNAs can post-transcriptionally regulate epigenetic-related genes and can act in the nucleus by stimulating or repressing gene transcription [81]. Post-transcriptional modifications can modulate protein level independently from correlating mRNA levels [82]. MicroRNAs regulate post-transcriptional epigenetic factors such as DNMTs, PRC1 and PRC2, heterochromatin protein 1 (HP1), and HDACs [81]. PRC1 and PRC2 are polycomb group proteins that cooperate with DNMTs to silence target genes [83]. HP1 proteins are critical mediators of heterochromatin gene silencing and gene activation [84]. Further, miRNAs stimulate or repress gene transcription. MicroRNAs can silence gene transcription with the use of AGO1, AGO2, and small interfering RNAs that recognize the target promoter region [85, 86]. MicroRNAs can target promoter elements and active RNA with the use of AGO1, AGO2, Drosha, and Dicer [87].

2.4 Female Reproduction

Multiple organs are required to facilitate healthy reproduction. Organs within the hypothalamic-pituitary-ovary axis (HPO) consist of the hypothalamus, anterior pituitary, and the ovary. The HPO axis facilitates healthy reproduction in mammals and normal development throughout the body. The hypothalamus, pituitary, and ovary secrete endogenous hormones critical for the differentiation, development and action of the brain, ovary, pituitary, reproductive tract, physiology, and behaviors [27, 88]. In adulthood, the HPO axis displays rhythmic patterns that vary from minutes to weeks depending on the organ and hormonal feedback between all of the organs [95].

Hypothalamus

The hypothalamus in mammals is a small region of the brain. It is near the base of the brain adjacent to the pituitary gland. In adulthood, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) to the pituitary as part of a feedback mechanism for reproductive functions. The hypothalamus contains two discrete regions critical for fertility: the anteroventral periventricular nucleus-periventricular nucleus continuum (AVPV/PeN) and the arcuate nucleus (ARC) [96]. Neurons in these regions express *Kiss1* genes, which encode for the neuropeptide kisspeptin [96]. Kisspeptin neuropeptides are proximal agonists of GnRH secretion from GnRH neurons [96, 97]. The hypothalamus secretes GnRH into the median eminence and then into the hypothalamo-hypophyseal portal circulation to the anterior pituitary [89]. GnRH is a hypothalamic peptide that mediates central control of reproduction. GnRH peptide release is pulsatile and the rhythmic nature of GnRH is critical for proper gonadotropin secretion from the anterior pituitary [98].

The secretion of GnRH in adult females is mediated by a feedback mechanism of gonadal sex steroid hormones. Specifically, the sex steroid hormones interact with neurons in the ARC to inhibit *Kiss1* expression, whereas the sex steroid hormones increase *Kiss1* expression in the AVPV [99]. The direct effect on *Kiss1* expression translates to GnRH secretion. The differential effects of sex steroid hormones on *Kiss1* secretion in the ARC and the AVPV translates the sex steroid hormone mediated positive and negative feedback mechanism of reproduction [96]. Specifically, the ARC is responsible for the negative sex steroid hormone feedback loop for GnRH secretion [100]. Conversely, the AVPV is responsible for the positive feedback effects of estrogen on GnRH secretion, and subsequently, the preovulatory LH surge [101].

Pituitary

The anterior pituitary consists of five different endocrine cell types: gonadotropes, lactotropes, somatotropes, thyrotrope, and adrenocorticotropes. The gonadotropes are endocrine cells that in response to GnRH pulses, produce gonadotropin peptides critical for reproduction [89]. The two gonadotropin peptides secreted by gonadotropes are follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both FSH and LH activate the gonads and are critical for normal steroidogenesis and gametogenesis. In females, LH stimulates ovulation as well as corpus luteum formation and function, whereas FSH stimulates ovarian follicle formation as well as estrogen secretion [89].

The pulse frequency of GnRH causes differential gonadotropin secretion by the gonadotropes. Specifically, increasing GnRH frequencies result in preferential secretion of LH and decreasing GnRH frequencies result in preferential FSH release [102]. The gonadotrope cells respond to GnRH through the GnRH receptor (GnRHR). The ability for the gonadotrope cell to

differentially respond to varying GnRH pulse frequencies may due to changes in the number of cell surface receptor numbers [102, 103].

The gonadotropin peptides, FSH and LH, are heterodimeric glycoprotein hormones that share a common α-glycoprotein hormone subunit (αGSU) and have distinct β subunits (LHβ and FSHβ) [89]. FSH consists of αGSU and FSHβ and LH is composed of αGSU and LHβ. The transcription of the β-subunits are dependent on the GnRH pulse frequency whereas αGSU is produced in excess and not dependent on GnRH pulse frequency [104]. However, the mechanism by which gonadotrope cells decode the pulsatile GnRH signal to produce either FSH or LH remains largely unknown [102].

Ovary

The ovary is a heterogeneous organ composed of follicles, oocytes, corpora lutea, and interstitial tissue. The primary roles of the ovary are to produce sex steroid hormones and maintain a steady stream of growing follicles. Normal sex steroid hormone production by the mature follicles is essential for the maintenance of normal menstrual/estrous cyclicity, reproductive tract tissues, and non-reproductive tissues such as bones, the heart, and the brain [105-108]. In addition, the mature antral follicle is the only follicle type capable of ovulating and releasing an oocyte for potential fertilization. Therefore, female fertility is dependent on the maintenance of a constant stream of growing follicles and sex steroid hormone secretion throughout the reproductive lifespan [109].

Primordial germ cell specification and migration

In mice, the ovary begins to form during embryonic development. Primordial germ cells are precursors of gametes that develop as sperm or eggs in the gonads. Primordial germ cells are first detected at embryonic day (ED) 6.0 – 6.5 by the exclusive expression of the protein

BLIMP1 [110]. Primordial germ cells are pluripotent stem cells that migrate during embryonic development to the place of gonadal development. During migration, the primordial germ cells undergo mitosis to increase primordial germ cell population. Primordial germ cells actively traverse through the developing embryo by receiving cues from the somatic cells, such as the chemokine SDF-1 [111]. At ED 10.5 in the mouse, a large population of primordial germ cells finish migration to the genital ridge and begin to form the ovary [112, 113]. The primordial germ cells continue to proliferate, but around ED 12, primordial germ cells enter meiosis and begin differentiating into germ cells [113]. Germ cells are connected by intracytoplasmic bridges and form large clusters. The germ cells begin to arrest in meiotic prophase and some germ cells begin to undergo apoptosis around ED 14.5 [112, 113]. The apoptotic process is necessary to eliminate abnormal or unhealthy germ cells unable to produce viable oocytes.

Germ cell nest breakdown

Following birth, pups experience a dramatic decrease in circulating estrogen and progesterone. The dramatic change of hormones initiates germ cell nest breakdown [114, 115]. Germ cell nest breakdown is the process that germ cells undergo apoptosis or differentiate into primordial follicles through a variety of signaling pathways such as the NOTCH2 signaling pathway [114, 116]. Only a third of the total germ cells survive to become primordial follicles [117, 118]. The primordial follicle consists of a single oocyte surrounded by a single layer of squamous somatic granulosa cells and subsequently becomes the finite pool of available follicles during the reproductive lifespan [113]. The B-cell lymphoma/leukemia-2 (Bcl-2) family of proteins is critical for the regulation of apoptosis in reproductive tissues and during germ cell nest breakdown. This family of proteins contains pro-apoptotic proteins and anti-apoptotic proteins. Proper regulation of these proteins leads to healthy and normal apoptosis during germ

cell nest breakdown [117, 119, 120]. Germ cell nest breakdown is complete around postnatal day 6 in the mouse [117]. Once the germ cell nest breaks down and individual germ cells are surrounded by somatic granulosa cells, the structure is a primordial follicle. Primordial follicles remain quiescent until activation through folliculogenesis [121].

Follicle recruitment, selection, and maturation

At one single time in the adult mouse ovary, all stages of follicle growth are present. Follicles are quiescent, constantly growing, or undergoing atresia. The manner in which a follicle remain quiescent, is recruited for growth, or undergoes atresia is a complex process. Specifically, primordial follicles are the most immature follicle type in the ovary. Primordial follicles may remain quiescent in the ovary and maintain the ovarian reserve through repressive signals. Specifically, phosphatase and tensin homolog (PTEN), a protein that antagonizes the phosphoinositide 3-kinase (PI3K) pathway, is critical to maintain primordial follicle dormancy [122]. It is critical to maintain primordial follicle dormancy so that primordial follicles may activate at different points throughout the reproductive lifespan of a female.

Primordial follicle may activate and grow into primary follicles through a variety of pathways. Specifically, proteins from the (transforming growth factor beta) TGFβ super family are critical to initiate follicle activation [123] and *in vitro* studies show that kit ligand, basic fibroblast growth factor, and bone morphogenetic protein 4 and 7 increase primary to primordial follicle ratio [124-127]. It is critical for primordial follicles to activate, grow, and potentially ovulate; however, studies show that the most common fate for ovarian follicles is atresia [17, 128]. A balance of pro- and anti-apoptotic factors regulates follicular atresia. The signaling pathway responsible for follicle death in the ovary includes the BCL2 family of proteins [117, 119, 120, 129], whereas the signaling pathways that help prevent follicle death include

gonadotropins, insulin-like growth factor-I, and estrogens [17, 130, 131]. Follicle atresia is important to remove unhealthy or poor quality oocytes from the germ cell pool.

Ovarian follicles mature through a process called folliculogenesis. Primordial follicles may activate and grow into primary follicles. Primary follicles are composed of a single oocyte surrounded by a single layer of cuboidal granulosa cells [121, 132]. Primary follicles then grow into preantral follicles, which consist of a single oocyte surrounded by at least two layers of cuboidal granulosa cells and a layer of thecal cells [121, 132]. Preantral follicles are composed of a single oocyte surrounded by numerous layers of granulosa cells, at least two layers of thecal cells, and a fluid filled space called the antrum [121, 132]. Preantral follicles subsequently grow into antral follicles. Antral follicles are the only follicle type that are capable of synthesizing sex steroid hormones such as progesterone, androgens, and testosterone and they are the only follicle type capable of ovulating for fertilization [133].

Gonadotropin signaling and ovarian follicles

The ovary receives gonadotropin signals from the pituitary. Ovarian follicles are stimulated by both FSH and LH to produce sex steroid hormones. Both FSH and LH bind to cell surface protein receptors and through complex signaling pathways coordinate gene expression critical for the many functions of the ovary [134]. Simply, FSH is critical for follicle maturation and LH is required for ovulation.

FSH regulates the expression of over 500 target genes in granulosa cells, which are somatic cells that support follicle maturation [135]. Relatively low, but constant levels of FSH are required to promote granulosa cell differentiation. FSH binds to the seven membrane-spanning G protein-coupled receptor, FSH receptor [134]. FSH signals and activates the PI3K pathway, which can signal for cell survival, granulosa cell differentiation and proliferation, and

promotes gene expression. Subsequently, FSH regulates apoptosis, proliferation, steroidogenesis, and promotes granulosa cells to respond to the LH surge largely through protein kinase A (PKA) [134]. FSH is critical for the growth and maturation of the ovarian follicles.

LH stimulates sex steroid hormone production in follicles, regulates thecal cell functions, and is critical for ovulation. LH enhances mRNA expression of transcription factors necessary for sex steroid hormone synthesis. Specifically, LH induces 17α-hydroxylase/C17-20-lyase (CYP17), which is the rate-limiting enzyme for androgen biosynthesis [136]. Further, LH signals through a pathway involving the orphan nuclear receptor NR4A1 to increase mRNA levels of steroidogenic acute regulatory protein (*Star*), cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1*), 3β-hydroxysteroid dehydrogenase (*Hsd3b1*), and cytochrome P450 family 17 subfamily A member 1 (*Cyp17a1*) in theca cells, which are enzymes necessary for sex steroid hormone synthesis [137].

Ovarian follicle steroidogenesis

In addition to follicle production and growth, the ovary is the major site of sex steroid hormone synthesis. Antral follicles are responsible for the primary production of estradiol because they contain the combination of theca cells, granulosa cells, and a series of enzymes [17]. Further, the corpus luteum is responsible for the primary production of progesterone because corpora lutea contain enzymes and luteal cells that specialize in progesterone production [138].

Within the antral follicle, the coordination between the theca and granulosa cells and signaling by FSH and LH are required for successful production of 17B-estradiol. Cholesterol is the precursor of all the sex steroid hormones produced in the ovary. The first conversion is of cholesterol to pregnenolone and it is the rate-limiting step subjected to acute regulation. The

protein StAR mediates the transfer of cholesterol from the outer mitochondrial membrane across the intercisternal space, and into the inner mitochondrial membrane of granulosa and theca cells [133, 139]. The enzyme CYP11A1 accesses cholesterol from the inner mitochondrial membrane and converts cholesterol to pregnenolone through three monooxygenations [140]. Pregnenolone diffuses from the mitochondria and transported to the smooth endoplasmic reticulum where it is further converted by CYP17A1 to dehydroepiandosterone (DHEA) or converted by HSD3B to progesterone. DHEA is catalyzed by HSD3B into androstenedione and progesterone is cleaved by CYP17A1 into androstenedione within the smooth endoplasmic reticulum. Androstenedione is a weak androgen that can be converted into a potent androgen, testosterone, by HSD17B [141] or aromatized by CYP19A1 into estrone [140]. Testosterone is aromatized by CYP19A1 and converted into 17B-estradiol, a potent estrogen [140]. Estrone is a weak estrogen that is converted by HSD17B into 17B-estradiol, [141].

Ovulation and corpora lutea formation and function

The corpus luteum is a transient organ that forms following follicle ovulation in preparation for pregnancy. The theca and granulosa cells left behind after ovulation luteinize to form a corpus luteum. Luteinization is composed of cell proliferation, cell differentiation, and tissue remodeling. Specifically, proliferation of theca and granulosa cells ceases and the cells undergo rapid hypertrophy and differentiate into luteal cells. In addition, during luteinization, blood vessels rapidly grow into the previously-granulosa layer of the corpus luteum [138, 142]. This highly vascular corpus luteum sequesters bloodborne sources of cholesterol as substrate for the steroidogenic pathway [138]. Luteinization of granulosa and theca cells increases the expression and/or activity of CYP11A1, the enzyme responsible for converting cholesterol or pregnenolone. Further, an increase in HSD3B expression and decrease in CYP17A1 and

CYP19A1 facilitates an increase of progesterone expression [138, 143]. The corpus luteum specializes in the production of progesterone to maintain a pregnancy following ovulation.

Reproductive senescence

Reproductive senescence is a normal process of reproductive aging that occurs in women. Over time, the ovarian reserve depletes and is the major contributor of reproductive senescence. The age of reproductive senescence in women is based on the size of the initial primordial follicle pool and the rate of follicle depletion. Normal ovarian functions decline and cease leading to a feedback cascade to the hypothalamus and pituitary [144]. The lack of estrogens and progesterone from the ovary removes the negative feedback on the hypothalamus and pituitary. Specifically, GnRH secretion increases and leads to an increase in serum FSH and LH levels. Additionally, the ovary does not produce inhibin and is also associated with dramatic increases of FSH and LH levels [145].

Uterus

The uterus is an organ critical for fertility in females. It functions as an endocrine sensitive organ that facilitates embryo implantation and delivery of the infant. The uterus is divided into three sections from anterior to posterior: the fundus, the corpus (body), and the isthmus (cervix). Further, the uterus is composed for three tissue layers: functionalis endometrium, basalis endometrium, and myometrium [108, 146]. The development of the uterus is complex, but simply, the paramesonephric or Müllerian ducts develop and give rise to the female reproductive organs through a series of growth pathways and hormone signals [147].

The three uterine tissue layers serve different purposes. The functionalis endometrium is the superficial tissue layer in the uterus that lines the lumen and it is sensitive to sex steroid hormones and critical for implantation. The functionalis endometrium, in response to sex steroid

hormones, undergoes dramatic changes in cell appearance and function throughout the menstrual and estrous cycle. The basalis endometrium is adjacent to the functionalis endometrium. This layer is critical for the regeneration of the functionalis endometrium following menses in women. The menstrual and estrous cycles are divided into two different phases. During the follicular or proliferative phase in women, the endometrium thickens approximately 12 – 14 mm due to the increasing levels of serum estradiol [108]. The endometrial glands also become larger and more tortuous and the stroma proliferates. The uterus is preparing for a pregnancy. During the follicular or proliferative phase in rodent models, the uterine width is at the greatest and endometrium growth occurs due to high levels of estrogen and low levels of progesterone [148]. During the luteal or secretory phase in women, the primary sex steroid hormone is progesterone. During this phase, the uterus enters a "window of receptivity" in which glycogen accumulates near the basalis endometrium and the epithelial cells of the endometrium expel proteins into the glandular lumen, endothelial cells proliferate, and edema occurs [149]. An embryo is supported by the endometrium during this period, however in women, if a pregnancy is not achieved, the endometrium undergoes apoptosis and the vascular basement membraned breaks down and the functionalis endometrium splits from the basalis endometrium and is discarded through the vagina [108, 150]. The women reenters the follicular or proliferative phase and the functionalis endometrium is regenerated by the basalis endometrium [151].

The myometrium contains smooth muscles and is the outer most layer of the uterus [146]. Similar to the endometrium, the myometrium is influence by sex steroid hormones. The myometrium is important for uterine contractions and aid in functions such as sperm transit, placement of the blastocyst to the luminal epithelium of the functionalis endometrium, contractions during menstruation, and parturition [108, 150, 152].

2.5 Epigenetic Effects of Endocrine Disruptors on Female Reproduction

The hypothalamus

Studies indicate that endocrine disrupting chemicals can target the hypothalamus in the brain, leading to epigenetic changes and transgenerational effects. For example, bisphenol A (BPA), a plasticizer with well-known endocrine disrupting effects, has been shown to cause transgenerational inheritance in the hypothalamus [90]. Prenatal BPA exposure reduces ERα-immunoreactivity in brain regions important for reproductive function in female mice in a transgenerational manner [90]. Further, perinatal BPA exposure increases the expression of *Meg3*, a maternally expressed lncRNA, in the female hypothalamus of the F3 generation [91]. The expression of *Meg3* is important because it is correlated with the central control of precocious puberty [92] and increased levels of BPA are associated with precocious puberty in women [93] and in laboratory animals [94]. The increase in *Meg3* is significant because it is an epigenetic modifier and mRNA expression is increased in the generation that was ancestrally exposed to BPA.

The ovary

Endocrine disruptors can affect several processes in ovary, including the formation of a healthy primordial follicle pool, maintenance of a constant stream of growing follicles, and normal steroidogenic capacity, all of which are required for normal female fertility [17, 109, 144, 153, 154]. Any chemical that interferes with these processes can cause severe reproductive outcomes. Specifically, chemicals that target the formation of the primordial follicle pool cause infertility because they deplete the finite follicle reserve used for the growth of ovulatory follicles [153, 154]. Additionally, an increased loss of primordial follicles leads to an early onset of reproductive senescence [144]. This is of concern because early onset of reproductive

senescence is associated with increased risk of chronic diseases [106, 144, 155-157]. Chemicals that specifically target primary, preantral, and antral follicles may lead to temporary infertility or permanent infertility. Temporary infertility may occur when the toxicant only targets the mature population of follicles, but not immature follicles. Thus, when the toxicant is removed, the immature follicle types can grow and replenish the mature population of follicles, restoring fertility. Permanent infertility occurs when the toxicant is not removed and continuously targets the growth and function of ovarian follicles [109]. Permanent infertility is more likely to occur in humans because chemical exposure occurs on a daily basis and it is difficult to remove chemical exposure. Chemicals that target the production of sex steroid hormones from the ovary may lead to infertility and other non-reproductive disorders [106, 109, 158-160].

Exposure to diethylstilbestrol (DES) has been associated with multigenerational effects on the ovaries in women. In particular, one case study describes small cell carcinoma of the ovary in a 15 year old girl whose maternal grandmother had taken DES during her pregnancy [161]. This study demonstrates that prenatal DES exposure is associated with a multigenerational increase in ovarian cancer in the F2 generation. Similarly, another study has shown that prenatal exposure to DES is associated with ovarian cancer in the F2 generation [162]. Unfortunately, the epigenetic mechanisms for these ovarian effects have not been fully investigated across generations.

Methoxyclor (MXC) is a banned insecticide that was once used as a replacement for dichlorodiphenyltrichloroethane (DDT). It is an endocrine disruptor shown to directly affect ovarian functions. Studies have shown the exposure to MXC causes various ovarian-related diseases in both multigenerational and transgenerational manners. Direct MXC exposure inhibits growth and induces atresia of antral follicles by altering the expression of regulators of apoptosis

and the cell cycle [163]. Additional evidence shows that MXC exerts these effects via the aryl hydrocarbon receptor [164]. Further, MXC exposure causes ovarian disease in the F1 generation of rats and ancestral MXC exposure increases polycystic ovarian-like syndrome in the F3 generation of rats [165]. Epigenetic analyses show that MXC hypermethylates CpGs in the ERβ promoter of the ovary. Further, MXC hypermethylates multiple loci critical for ovarian signaling pathways and concurrently decreases gene expression [166, 167]. MXC exposure also increases expression of DNMT3B in the ovaries, suggesting that DNMT3B plays a critical role in DNA hypermethylation [166].

Phthalates are a class of chemicals commonly used as plasticizers, but are also known endocrine disrupting chemicals. Previous studies show that prenatal exposure to a mixture of phthalates that mimics human exposure causes multigenerational effects on mouse ovaries [168, 169]. Specifically, prenatal phthalate exposure induces cystic ovaries in the F1 and F2 generations [168, 169]. Further, exposure to a mixture of plastic derivatives (BPA, di(2-ethylhexyl) phthalate, and dibutyl phthalate) causes polycystic ovaries in both the F1 and F3 generations [170]. Exposure a single phthalate, di(2-ethylhexyl) phthalate (DEHP), also causes adverse transgenerational effects on the ovary in mice. Specifically, prenatal DEHP exposure dysregulates folliculogenesis, alters sex steroid hormone levels, and increases the presence of ovarian cysts in a multigenerational manner [171, 172]. Further, ancestral exposure to DEHP accelerates early folliculogenesis in a transgenerational manner [172]. Although studies demonstrate that phthalate exposure causes transgenerational effects on the ovary, the mechanisms causing these effects are not well understood. A few studies suggest that DEHP exposure causes multigenerational effects through both the estrogen receptor 1 (ESR1) and the

peroxisome proliferator-activated receptor alpha (PPARα) [173-176]. However, further studies are necessary to investigate the effects of phthalate exposure on the epigenome of the ovary.

BPA, a plasticizer with known endocrine disrupting abilities, causes both multigenerational and transgenerational effects on the ovary. Prenatal exposure to BPA decreases serum testosterone levels in the F2 generation and dysregulates steroidogenic enzymes in the F2 ovaries of mice [177]. Ancestral exposure to BPA dysregulates gene expression of ovarian apoptotic factors, oxidative stress factors, and autophagy factors in mice [178]. Interestingly, a few studies have linked BPA exposure to DNA methylation across generations in females [170, 179]. Specifically perinatal exposure to BPA altered DNA methylation at a differentially methylated region that regulates expression of *Igf2* gene in F1 and F2 generations, however, this was found only in male mice [179]. Another study has demonstrated that prenatal exposure to a mixture of BPA and phthalates promotes epigenetic transgenerational inheritance of disease. However, this study only showed that the plasticizer mixture affected the differentially methylated regions in sperm. Epigenetic analyses were not performed on the females [170].

Vinclozolin, a fungicide used on fruits, acts as an endocrine disruptor and causes transgenerational effects [180, 181]. Prenatal vinclozolin decreases primordial follicle counts in both the F1 and F3 generations of rats at one year of age [181]. In addition, ancestral exposure to vinclozolin causes small and large cysts in the ovaries and increases circulating androstenedione levels in the F3 generation [181]. The observed phenotype in the F3 generation is similar to the clinical phenotype in women with polycystic ovarian syndrome [181]. Further, vinclozolin causes differential gene expression in the F3 ovaries. These genes are associated with ovarian diseases such as polycystic ovarian syndrome. Ancestral vinclozolin exposure also alters DNA methylated regions in promoter regions of the granulosa cells; however, the DNA methylation

changes do not overlap with the promoters of the differential gene expression in the F3 generation [181]. Instead, the DNA methylation changes may influence distal gene expression through ncRNA, which may regulate the differential gene expression in the granulosa cells of the F3 ovaries [181]. Further investigations reveal that the purified rat granulosa cells from ancestrally exposed F3 generation have differentially expressed lncRNA and sncRNAs and that these changes contribute to the vinclozolin-induced dysregulation of the ovary [182].

Although studies demonstrate that environmental chemicals can induce transgenerational effects on the ovary, diet has also been shown to cause endocrine disruption and alter the epigenome in the ovary. Specifically, exposure to low-protein diet during pregnancy in the F0 generation accelerates ovarian aging in a multigenerational manner (F1-F2 generations) in female rats. Exposure to the low-protein diet during pregnancy reduces ovarian telomere length, primordial follicle numbers, and serum anti-Müllerian hormone levels in the F1 and F2 generation rats [183]. These reductions in the F1 and F2 generations are due to an increase in oxidative stress markers such as increased mitochondrial DNA copy numbers and 4-hydroxy-nonenal levels in rat ovaries [183]. Unfortunately, epigenetic modifications were not quantified in this study [183]. However, a different study examined the transgenerational effects of supplementing polyunsaturated fatty acids (PUFAs) in the diet of female mice from the F0 – F3 generations [184]. Exposure to n-3 PUFAs reduced the number of ovulated oocytes, increased the percentage of oocytes trapped in luteinized follicles, and decreased litter sizes in the F3 generation [184]. Further, increased PUFA exposure decreased concentrations of prostaglandins in the F3 generation [184].

Another study examined the transgenerational effects of a restricted diet during pregnancy on epigenetic markers in the offspring [185]. In this study, the F0 rats were fed half of

the typical food intake during pregnancy. The rats with prenatal restricted diet exposure exhibited changes in epigenetic markers across the generations. Specifically, mRNA levels of *Hdac1*, a histone modification gene, were upregulated in the F1 and F2 generations and downregulated in the F3 generation [185]. DNA methylation specific genes such as *Dnmt1* was altered across the generations. Although these epigenetic changed were not specifically observed in the ovary, it is worth noting that poor diet during pregnancy causes epigenetic changes that are inherited in the maternal germline, causing a transgenerational phenotype [185].

The uterus

The uterus is critical for fertility in females; it acts as an endocrine sensitive organ that facilitates both embryo implantation and parturition. Studies indicate that endocrine disruptors can affect the uterus and that these changes may lead to epigenetic and transgenerational inheritance of diseases. The prescription of diethylstilbestrol (DES) to pregnant women is one of the best examples of multi- and transgenerational impact because it is associated with fetal endocrine disruption and adverse reproductive health outcomes in subsequent generations in humans [186]. Women who were exposed to DES as a fetus, also known as “DES daughters”, have more frequent benign reproductive tract problems, including reproductive organ dysfunction, abnormal pregnancies, structural changes of the uterus, and reduced fertility [187]. These women have an increased risk of a rare clear-cell cervicovaginal adenocarcinoma and squamous-cell and cervicovaginal carcinoma [188]. Further, these “DES daughters” report that their *in utero* exposure led to cancer in their daughters, the F2 generation [189]. Effects seen in this F2 generation demonstrate a multigenerational effect of prenatal DES exposure on the uterus in humans [189]. Animal studies further demonstrate multigenerational effects of DES. Specifically, prenatal DES exposure decreases fertility in the F1 generation of female mice and

increases the incidence of malignant reproductive tract tumors such as adenocarcinomas in the F2 generation of female mice [190]. Additional studies show that prenatal and perinatal exposure to DES increases the susceptibility of uterine developmental abnormalities and cancer in both the F1 and F2 generations of female mice [191].

Currently, the mechanisms explaining the multigenerational effects of DES exposure on the uterus are not understood. However, studies in mice suggest epigenetic alterations in DNA methylation involving hormone responsive families of genes including lactoferrin, homeobox, wnt signaling pathway, and epidermal growth factor genes are involved with the reproductive tract developmental changes in a multigenerational manner [162, 192]. Another study demonstrates that neonatal DES exposure alters the expression of chromatin-modifying proteins in the adult mouse uterus, causing persistently altered epigenetic marks [193]. Further, neonatal DES exposure also decreases *Dnmt* gene expression and alters DNA methylation in the mouse uterus [194]. Although these epigenetic markers are observed from neonatal exposure within the same generation and not from prior generation exposures, these epigenetic changes may help contribute to the multigenerational effects of DES exposure.

Another endocrine disruptor that targets the uterus is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD is a byproduct of incomplete combustion of a variety of products such as fossil fuels, wood, and industrial wastes. Perinatal TCDD exposure causes endometriosis-like reproductive phenotypes in F1 – F4 generations of female mice, indicating both multi- and transgenerational effects [195]. Further, TCDD exposure reduces uterine progesterone responsiveness and causes subfertility [195]. TCDD exposure also causes endometriosis-like histological and functional phenotypes in mice [195]. TCDD exposure increases both stromal cell and epithelial cell ERS2 protein expression in the F1 – F3 generations and causes

adenomyosis in the F3 generation [196]. Interestingly, ancestral exposure to TCDD causes hypermethylation of *Pgr*, which is associated with the development of the endometriosis-like histological and functional phenotypes [197].

Female reproduction outcomes

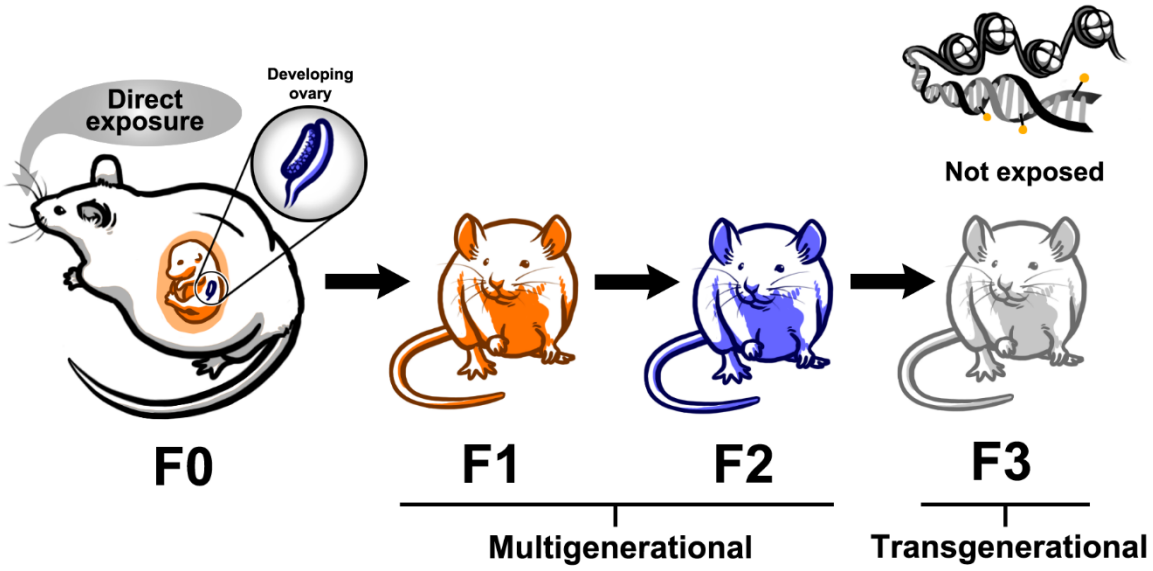
Plasticizers that act as endocrine disruptors interfere with female reproductive outcomes. Some of these reproductive outcomes are due to impacts on the hypothalamus, pituitary, ovary, and uterus. For example, prenatal BPA exposure reduces the ability of mice to maintain pregnancies in a multigenerational manner and it delays the onset of puberty and compromises the ability of mice to become pregnant in a transgenerational manner [198]. BPA has been shown to interfere with brain and ovarian functions in a transgenerational manner, likely contributing to altered reproductive outcomes [91, 198]. A study of offspring to Chinese textile workers shows that *in utero* serum dichlorodiphenyldichloroethylene (DDE) levels were associated with an early menarche in the offspring [199]. Both prenatal and ancestral exposure to a mixture of phthalates cause pregnancy complications in the F2 and F3 generations of mice [168]. Further, ancestral exposure to a phthalate mixture reduces total litter size and the percentage of dams that produce live litters in the F3 generation [168], whereas exposure to DEHP, a single phthalate, causes multi- and transgenerational effects on reproductive outcomes [172, 200]. Specifically, prenatal DEHP exposure increases litter size and decreases the percentage of dams that give birth in the F2 generation [200]. Ancestral DEHP exposure accelerates the onset of puberty and reproductive senescence in the F3 generation of female mice [171, 200]. Phthalates interfere with ovarian functions and may contribute to these altered reproductive outcomes [168, 171, 172, 200, 201].

2.6 Conclusions

Overall, the literature shows that several endocrine disrupting chemicals cause reproductive dysfunction in females in a multigenerational and transgenerational manner and that some of these effects are due to epigenetic changes. Epidemiological data show that exposure to endocrine disruptors is associated with adverse ovarian and uterine health outcomes in women across generations [161, 189, 199]. Experimental data demonstrate that endocrine disruptors cause female reproductive abnormalities in the hypothalamus, ovary, and uterus in multigenerational and transgenerational manners [91, 165, 168, 171, 172, 190, 200, 202]. Generally, the consensus is that epigenetic changes are induced by chemical exposures and are inherited through the germline, thus causing transgenerational phenotypes in reproductive functions in the generation that was not directly exposed to the endocrine disruptor. However, it is critical that future studies continue to investigate the epigenetic basis of transgenerational inheritance and demonstrate that the epigenetic changes are inherited through the germline. It is crucial to fill the gap in knowledge about how endocrine disruptors affect the epigenome so that potential interventions can be developed and used to stop endocrine disruption of female reproductive health.

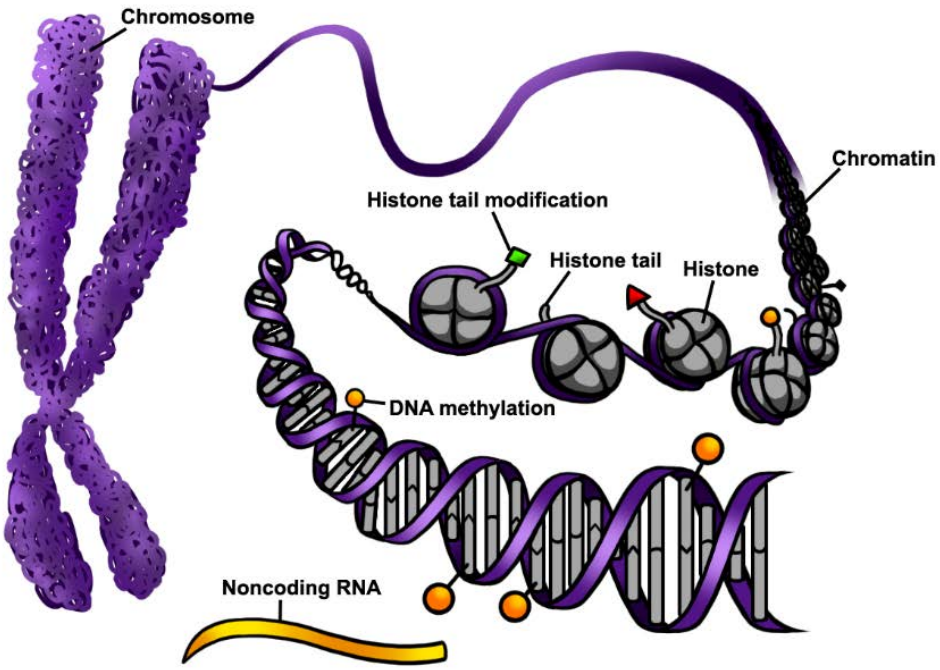
2.7 Table, Figures, and Legends

Figure 2.1 Multigenerational and transgenerational effects



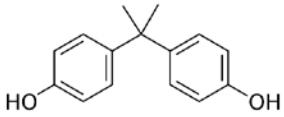
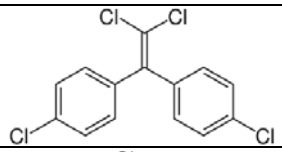
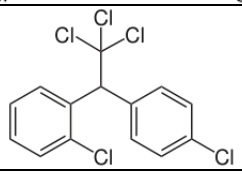
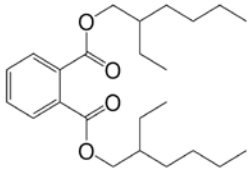
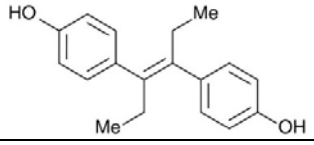
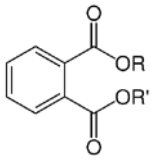
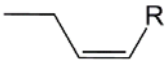
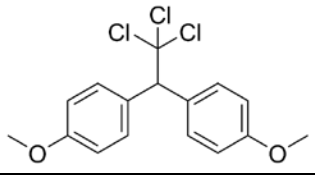
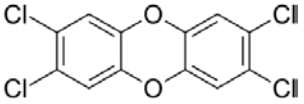
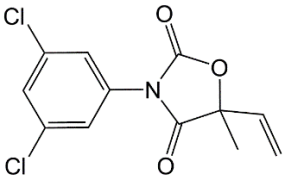
Exposure to endocrine disruptors during prenatal development causes multigenerational effects in the F1 and F2 generations and transgenerational effects in the F3 generation. The F1 and F2 generations are directly exposed to the endocrine disruptor as a fetus and germ cell, respectfully. The F3 generation is not directly exposed and the mechanisms governing the effects in the F3 generation are thought to be epigenetic in nature.

Figure 2.2 Epigenetic mechanisms



An overview of epigenetic mechanisms including chromatin modifications, DNA methylation, and noncoding RNA interactions. Chromatin is made of DNA, histone proteins, and nucleosomes and regulates gene expression by controlling the access of transcription factors to DNA. DNA methylation creates a physical barrier that generally impedes transcription factor binding. Noncoding RNA interact with DNA in many ways as a form of epigenetic modification.

Table 2.1 Endocrine disruptors and their chemical structures

Endocrine Disruptor Name	Chemical Structure
Bisphenol A (BPA)	
Dichlorodiphenyldichloroethylene (DDE)	
Dichlorodiphenyltrichloroethane (DDT)	
Di(2-ethylhexyl) phthalate (DEHP)	
Diethylstilbestrol (DES)	
Phthalates	
n-3 Polyunsaturated fatty acids (PUFAs)	
Methoxyclor (MXC)	
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	
Vinclozolin	

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CHAPTER 3

Prenatal Exposure to Di(2-ethylhexyl) Phthalate Disrupts Ovarian Function in a Transgenerational Manner in Female Mice[1]

3.1 Abstract

Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer found in polyvinyl chloride products such as vinyl flooring, plastic food containers, medical devices, and children's toys. DEHP is a ubiquitous environmental contaminant and is a known endocrine disrupting chemical. Little is known about the effects of prenatal DEHP exposure on the ovary and whether effects occur in subsequent generations. Thus, we tested the hypothesis that prenatal exposure to DEHP disrupts ovarian functions in the F1, F2, and F3 generations of female mice. To test this hypothesis, pregnant CD-1 mice were orally dosed with corn oil (vehicle control) or DEHP (20 and 200 µg/kg/day and 200, 500, and 750 mg/kg/day) daily from gestation day 10.5 until birth (7-28 dams/treatment group). F1 females were mated with untreated males to obtain the F2 generation, and F2 females were mated with untreated males to produce the F3 generation. On postnatal days 1, 8, 21, and 60, ovaries were collected and used for histological evaluation of follicle numbers and sera were used to measure progesterone, testosterone, 17β-estradiol, luteinizing hormone, and follicle stimulating hormone levels. In the F1 generation, prenatal exposure to DEHP disrupted body and organ weights, decreased folliculogenesis, and increased serum 17β-estradiol levels. In the F2 generation, exposure to DEHP decreased body and organ weights, dysregulated folliculogenesis, and disrupted serum progesterone levels. In the F3 generation, DEHP exposure accelerated folliculogenesis. These data suggest that prenatal exposure to DEHP leads to adverse multigenerational and transgenerational effects on ovarian function.

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3.2 Introduction

Phthalates are a family of synthetic chemicals that are additives and act as plasticizers to confer flexibility and prevent plastics from becoming brittle [1]. Many types of phthalates exist, but di(2-ethylhexyl) phthalate (DEHP) is a common plasticizer found in polyvinyl chloride products such as consumer products, medical equipment, and building products [2]. DEHP is incorporated into a multitude of products including personal care products, medical equipment (i.e., blood and I.V. bags), car upholstery, food and beverage containers, and building materials [3]. On average, 300 million pounds of DEHP are produced annually in the United States [1]. DEHP is noncovalently bound; therefore, it readily leaches from products and into the environment after repeated use, heating, and cleaning [4]. Humans are exposed to DEHP by oral ingestion, inhalation, and dermal contact at an average range between 3 – 30 μg/kg/day [3, 5]. Studies show that 100% of human urine samples test positively for DEHP and its metabolites, indicating that humans are repeatedly and continuously exposed to DEHP [6]. This is supported by the frequent detection of DEHP in various human tissues such as blood, amniotic fluid, umbilical cord blood, breast milk, and ovarian follicular fluids in humans [3, 7-10]. This is of concern because a pregnant mother and her developing offspring can be exposed to DEHP, and DEHP is a known endocrine disrupting chemical (EDC) [1].

Numerous studies demonstrate that exposure to DEHP exerts adverse effects on the male reproductive system. Specifically, prenatal exposure to DEHP causes testicular dysgenesis syndrome [11-14], which is characterized by testicular cancer, low or declining semen quality, high frequencies of undescended testis, and hypospadias [15, 16]. In addition, prenatal exposure to DEHP shortens anogenital distance (an indicator of *in utero* sex steroid hormone level

disruption) [17], decreases circulating serum testosterone levels [13], and causes pubertal abnormalities [18].

In females, the effects of prenatal exposure to DEHP are less understood than in males. However, experimental studies show that prenatal exposure to DEHP decreases litter size and pup body weight [19], disrupts sex determination, causes precocious puberty in females [20], and decreases circulating estradiol levels in females [21]. Further, studies indicate that prenatal exposure to DEHP increases the time for females to become pregnant and increases pup death in mice [2] and that it is associated with reduced uterine size [22], reduced testosterone:estradiol ratio and progesterone levels [23], and advanced age of pubic hair development in young girls [24].

Studies have examined the effects of DEHP exposure on the ovary because it is an important organ for reproductive processes. Further, a previous study has shown that DEHP metabolites reach the ovary [25]. Specifically, the bioactive metabolite of DEHP, mono(2-ethylhexyl) phthalate (MEHP), has been detected at approximately 9.34 ng/mL in human antral fluid [25].

The ovary is a heterogeneous organ composed of different follicle types, oocytes, corpora lutea, and interstitial tissue. Any chemical exposure that interferes with the development and function of the ovary can cause severe reproductive abnormalities. Specifically, chemicals that target the formation of the primordial follicle pool cause infertility because it depletes the finite follicle reserve used for the production of ovulatory follicles [26, 27]. Chemicals that specifically target primary, preantral, and antral follicles may lead to temporary infertility or permanent infertility. Permanent infertility may occur when the toxicant is not removed and it continuously targets the growth and function of ovarian follicles [28]. Chemicals that target the antral follicles

also may interfere with the production of sex steroid hormones, leading to infertility and other reproductive disorders [28-32]. Our laboratory has previously shown that *in vitro* exposure to MEHP reduces follicle growth in antral follicles and decreases sex steroid hormone biosynthesis [33]. Further, we have shown that DEHP exposure (20, 200, and 750 mg/kg/day) during adulthood for 10 days accelerates primordial follicle recruitment [34] and reduces the primordial follicle pool at 9 months [27]. In addition, studies indicate that postnatal exposure to DEHP (20 and 40 μg/kg) reduces the ovarian primordial follicle pool, accelerates ovarian follicular recruitment [19], and decreases ovarian concentrations of progesterone, 17β-estradiol, and androstenedione [35].

Although the direct effects of DEHP are fairly well documented on the ovary, the effects of DEHP exposure across generations are not as well understood. Previous studies have shown that maternal DEHP exposure (0.05 mg/kg/day) reduced embryo viability over several generations in female mice [36] and prenatal exposure to a mixture of plasticizers, including DEHP, increased the presence of small and large cysts in the ovary [37].

Although previous studies have shown that DEHP exposure adversely affects the ovary, they have not assessed the impact of prenatal DEHP exposure on follicle numbers in detail, sex steroid hormone production over time, and the impact of DEHP on the ovary in subsequent generations over time. Therefore, the current study was designed to evaluate the potential effects of prenatal exposure to DEHP during the second half of pregnancy on ovarian functions in the F1, F2, and F3 generations of mice. Specifically, this study tested the hypothesis that prenatal DEHP exposure adversely affects folliculogenesis, gonadotropin hormone levels, and sex steroid hormone levels in female mice over several generations.

3.3 Materials and Methods

Chemicals

DEHP (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of DEHP (0.022, 0.224, 560, and 840 mg/mL) were prepared by diluting the chemical in tocopherol-stripped corn oil (MP Biomedicals, Solon, OH). These stock solutions were diluted to achieve the desired doses of 20 µg/kg/day, 200 µg/kg/day, 200 mg/kg/day, 500 mg/kg/day, and 750 mg/kg/day. DEHP concentrations were chosen based on previous studies and their environmental relevance [2, 12, 26, 27, 33, 34]. Specifically, DEHP at 20 µg/mg/day was selected based on the United States Environmental Protection Agency published reference safe dose (https://www3.epa.gov/airtoxics/hlthef/eth-phth.html) and falls within the estimated human exposure range based on urinary metabolite levels [5]. DEHP at 200 µg/mg/day was used because adult exposure causes abnormal estrous cyclicity and accelerates primordial follicle recruitment in female CD-1 mice [34]. DEHP 200 mg/kg/day was chosen because adult exposure has been shown to accelerate primordial follicle recruitment in females [34]. DEHP 500 mg/kg/day was selected because this dose causes abnormalities in spermatogonial stem cells across multiple generations in male CD-1 mice [12]. DEHP 750 mg/kg/day was selected because it causes in abnormal estrous cyclicity and accelerates primordial follicle recruitment in adult female CD-1 mice [34].

Animals and study design

Adult female and male CD-1 mice (Charles River, USA) were housed at 25 °C in conventional polysulfone, ventilated cages on 12L:12D cycles. The mice were provided Teklad Rodent Diet 8604 (Harlan) and highly purified water (reverse osmosis filtered) in polysulfone

water bottles *ad libitum*. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and abide by the guidelines set forth by the National Institute of Health for the Care and Use of Laboratory Animals. Investigations using experimental animals or subjects were conducted in accordance with the Society for the Study of Reproduction's specific guidelines and standards.

At 8 weeks of age, 83 female mice (F0) were mated with control mice of the same age. The female mice were monitored twice a day for the presence of a copulatory vaginal sperm plug to confirm mating. Once a copulatory vaginal sperm plug was confirmed, the presence of which was considered gestational day (GD) 0.5, the females were weighed and individually housed. Subsequently, mice were weighed twice a week to confirm successful pregnancy. From GD 10.5 until birth of the pups, pregnant dams (F0) were orally dosed once a day with the vehicle control (tocopherol-stripped corn oil), or with DEHP (20 µg/kg/day, 200 µg/mg/day, 200 mg/kg/day, 500 mg/kg/day, 750 mg/kg/day) by placing a pipette tip with the dosing solution into the cheek pouch of the mouse. This dosing regimen was selected to mimic oral exposure to DEHP in humans [1, 27, 34]. The doses were calculated and adjusted based on daily body weights, and delivered in 25 – 33 µL of tocopherol-stripped corn oil. The treatment window was chosen because it is a critical time period of ovarian development. Specifically, this is when primordial germ cells arrive at the gonad [38, 39], sex determination occurs [40], and when global demethylation and imprint erasure of primordial germ cells occur [41].

Pregnant mice were allowed to deliver naturally and the day of birth was considered postnatal day (PND) 0. Mice born from the F0 generation were labeled the F1 generation. Female mice from the F1 generation were mated with non-treated male CD-1 mice at 3 months of age to produce the F2 generation. Females from the F2 generation were mated with non-

treated male CD-1 mice at 3 months of age to produce the F3 generation. The 200 mg/kg/day treatment group was not continued past the F2 generation. Body weights in all generations were measured from PND 1 – 60, and at 3, 6, and 9 months of age during the collection of organs and during set intervals during adulthood. Mice were euthanized on PNDs 1 and 8 by decapitation, and on PNDs 21 and 60 by carbon dioxide asphyxiation followed by cervical dislocation.

Histological evaluation of follicle numbers

Ovaries from F1, F2, and F3 females at PND 1, 8, 21, and 60 were collected, oviducts and surrounding tissues were removed, weighed in pairs, and placed in Dietrich's fixative. Fixed ovaries were placed in paraffin and serial sectioned at 5 µm for PND 1 ovaries or at 8 µm for PND 8, 21, and 60 ovaries. The sections then were mounted on glass slides, stained with Weigert's hematoxylin and picric-acid methyl blue, and covered with a glass coverslip. Every 5^{th} section of the ovary at PND 1 or every 10^{th} section of the ovary at PND 8, 21, and 60, was used to count the numbers of germ cells, primordial follicles, primary follicles, preantral follicles, antral follicles, and atretic follicles as described [2, 42]. Germ cells were defined as round in appearance with a nucleus. Primordial follicles were defined as follicles with an oocyte, surrounded by a single layer of squamous granulosa cells. Primary follicles consisted of an oocyte, surrounded by a single layer of cuboidal granulosa cells. Preantral follicles contained an oocyte, surrounded by multiple layers of cuboidal granulosa cells, and theca cells. Antral follicles consisted of an oocyte, surrounded by numerous layers of cuboidal granulosa cells, theca cells, and a fluid filled antrum. Preantral and antral follicles were only counted if they contained a nucleus in the oocyte to avoid "double counts" due to the large follicle size that can span multiple sections. Follicles that were transitioning between stages were counted as the more

immature stage. Atretic follicles were counted as preantral or antral follicles that contained 10% or greater number of apoptotic bodies. The total numbers of all follicles, total numbers of each healthy follicle type, percentages of follicle types, and percentages of atretic preantral and antral follicles were recorded. To calculate the percentages of follicle types, the number of the follicle type was divided by the total number of all follicles multiplied by 100. This calculations allow us to identify shifts in follicle pools, which may indicate either accelerated or inhibited folliculogenesis. To calculate the percentage of atretic follicles, the number of atretic preantral and atretic antral follicles were combined, divided by the number of healthy preantral and antral follicle types, and multiplied by 100. Corpora lutea were quantified at PND 60 by inspecting the individual progression of the corpora lutea throughout the ovary in all serial sections [43]. This was done to avoid double counting of corpora lutea because they do not have a landmark such as nuclear material in the oocyte observed in antral follicles. All sections were examined without knowledge of treatment group.

Analysis of sex hormone levels

Tissues and sera were collected and analyzed as described below. Mice do not cycle at PNDs 1 – 21; therefore, serum samples were collected at exactly 1, 8, or 21 days. Mice at PND 60 are cycling; therefore, serum samples were collected when the mice were in diestrus/metestrus to minimize fluctuations due to cycle day. All serum samples were submitted to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core lab without knowledge of treatment groups to measure progesterone, testosterone, and 17β-estradiol using enzyme-linked immunosorbent assays (ELISAs) and to measure follicle stimulating hormone (FSH) and luteinizing hormone (LH) using radioimmunoassay (RIAs). The

lowest limit of detections were 0.15 ng/mL for progesterone, 10 ng/dL for testosterone, 3 pg/mL for 17β-estradiol, 1.17 ng/mL for FSH, and 0.04 ng/mL for LH. If the assays measured less than the lowest limit of detection, the value was substituted with the lowest limit of detection/√2. The intra- and inter-assay coefficients of variability were less than 10% (https://med.virginia.edu/research-in-reproduction/wp-content/uploads/sites/311/2016/08/2016-INTRA-INTER-ASSAY-CVs__032316.pdf). At PND 21, low quantities of serum prevented measurements of serum progesterone levels for the F1 generation.

Statistical analyses

Data were expressed as the mean ± standard error of the mean (SEM). In all generations, data from multiple female pups originating from the same litter were averaged and combined as n = 1, and data from at least 3 separate litters were used in the analyses. If samples were less than 3, then statistical tests were not performed, but data were presented. Data were analyzed by comparing treatment groups to control using SPSS software (SPSS Inc., Chicago, IL). Outliers were removed using the GraphPad outlier calculator for the Grubb's test (GraphPad Software Inc., La Jolla, CA). To test for cohort differences, data were tested using a general linear model univariate test. If there was an interaction effect between treatment and cohort in the tests of between-subjects effects, then the data from the first two cohorts were analyzed. If no interaction effect between treatment and cohort occurred, then all three cohorts were analyzed as one. Data that were continuous were assessed for normal distribution by Shapiro-Wilk analysis. If data met assumptions of normal distribution and homogeneity of variance, data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett (2-sided) *post-hoc* comparisons. If data were not normally distributed, were presented as a percentage, and/or did not meet homogeneity

of variance assumptions, the independent sample Kruskal-Wallis H followed by Mann-Whitney U non-parametric tests were performed. For all comparisons, statistical significance was determined by a p-value ≤ 0.05. In instances in which p-values were greater than 0.05, but less than 0.1, data were considered to exhibit a trend towards significance.

3.4 Results

Effects of DEHP exposure on body weights

In the F1 generation, DEHP exposure did not significantly affect body weight at any time point (Table 3.1). In the F2 generation, exposure to the 20 µg/kg/day dose of DEHP significantly decreased body weight at PNDs 8 and 21 compared to the control (Table 3.1, n = 7 – 20 dams/treatment group; $p \leq 0.05$), but DEHP did not affect body weights at other time points. In the F3 generation, DEHP exposure did not significantly affect body weight at any time points (Table 3.1).

Effects of DEHP on ovarian weights

In the F1 generation, the 20 µg/kg/day and 750 mg/kg/day doses of DEHP significantly decreased ovarian weight, but the 200 µg/kg/day dose of DEHP significantly increased ovarian weight at PND 21 compared to the control (Table 3.2, n = 3 – 15 dams/treatment group; $p \leq 0.05$). Exposure to the 200 mg/kg/day dose of DEHP increased ovarian weight at PND 21, but this increase only trended towards significance compared to the control (Table 3.2, n = 8 – 15 dams/treatment group; $p = 0.076$). In the F2 generation, exposure to the 20 µg/kg/day dose of DEHP decreased ovarian weight at PND 21, but this decrease only trended towards significance (Table 3.2, n = 8 – 9 dams/treatment group; $p = 0.072$). Exposure to the 200 µg/kg/day dose of

DEHP significantly decreased ovarian weight at PND 60 compared to the control (Table 3.2, n = 7 – 9 dams/treatment group; $p \leq 0.05$). In the F3 generation, DEHP exposure did not significantly affect ovarian weight at any time points (Table 3.2).

Effects of DEHP on uterine weights

In the F1 generation, exposure to DEHP did not significantly affect uterine weight at PND 8, but the 200 µg/kg/day dose of DEHP significantly increased uterine weight at PND 21 compared to the control (Table 3.3, n = 4 – 16 dams/treatment group; $p \leq 0.05$). In the F2 generation, exposure to the 500 mg/kg/day dose of DEHP significantly increased uterine weight at PND 8 (Table 3.3, n = 3 – 14 dams/treatment group; $p \leq 0.05$). In contrast, the 200 mg/kg/day dose of DEHP decreased uterine weight at PND 8, but only trended towards significance (Table 3.3, n = 7 – 14 dams/treatment group; $p = 0.084$). At PND 21, exposure to the 20 µg/kg/day dose of DEHP significantly decreased uterine weight compared to the control (Table 3.3, n = 7 – 15 dams/treatment group; $p \leq 0.05$). In the F3 generation, exposure to DEHP did not significantly affect uterine weight at any of the time points (Table 3.3).

Effects of DEHP on liver weights

In the F1 generation, the 500 mg/kg/day dose of DEHP significantly increased liver weight at PND 1 compared to the control (Table 3.4, n = 5 – 17 dams/treatment group; $p \leq 0.05$) and the 200 mg/kg/day dose of DEHP significantly increased liver weight at PND 8 compared to the control (Table 3.4, n = 8 – 15 dams/treatment group; $p \leq 0.05$). In the F2 generation, the 500 mg/kg/day dose of DEHP significantly increased liver weight at PND 8 compared to the control (Table 3.4, n = 10 – 17 dams/treatment group; $p \leq 0.05$). The 20 µg/kg/day dose of DEHP

significant decreased liver weight, and the 500 mg/kg/day dose of DEHP significantly increased liver weight at PND 21 compared to the control (Table 3.4, n = 3 – 15 dams/treatment group; $p \leq 0.05$). In F3 generations, exposure to DEHP did not significantly affect liver weight at any doses (Table 3.4).

Effect of DEHP exposure on F1 ovarian morphology

At PND 1, prenatal exposure to DEHP did not significantly affect the numbers of germ cells, or primordial, primary, and total follicles compared to the control (Figure 3.1A). Similarly, at PND 8, DEHP exposure did not significantly affect the numbers of primordial, primary, preantral, or total follicles compared to the control (Figure 3.1B). In contrast, at PND 21, the 200 µg/kg/day and 750 mg/kg/day doses of DEHP significantly decreased the numbers of antral follicles compared to the control (Figure 3.1C, n = 4 – 10 dams/treatment group; $p \leq 0.05$). At PND 60, both the 20 µg/kg/day and the 200 µg/kg/day doses of DEHP decreased the number of primary follicles, but only trended towards significance (Figure 3.1D, n = 5 – 13 dams/treatment group; p = 0.071 and 0.064, respectfully). Further, the 20 µg/kg/day dose of DEHP decreased the number of preantral follicles but only trended towards significance (Figure 3.1D, n = 5 – 13 dams/treatment group: p = 0.084).

At PND 1, prenatal exposure to DEHP did not significantly affect the percentage of germ cells, or primordial, primary, and atretic follicles compared to the control (Figure 3.2A). In contrast, at PND 8, exposure to the 500 mg/kg/day dose of DEHP increased the percentage of primordial follicles and the 200 mg/kg/day dose of DEHP increased the percentage of preantral follicles, but both increases trended towards significance compared to the control (Figure 3.2B, n = 5 – 14 dams/treatment group; p = 0.085 and p = 0.076, respectfully). At PND 21, the 20

μg/kg/day dose of DEHP significantly increased the percentage of primary follicles and the 750 mg/kg/day dose of DEHP significantly decreased the percentage of antral follicles compared to the control (Figure 3.2C, n = 5 – 12 dams/treatment group; $p \leq 0.05$). Further, the 500 mg/kg/day dose of DEHP decreased the percentage of antral follicles, but the decrease only trended towards significance (Figure 3.2C, n = 5 – 12 dams/treatment group; p = 0.079). At PND 60, the 200 μg/kg/day dose of DEHP decreased the percentage of primary follicles, but the decrease only trended towards significance (Figure 3.2C, n = 5 – 13 dams/treatment group; p = 0.068). Further, the 200, 500, and the 750 mg/kg/day doses of DEHP significantly decreased the percentage of atretic follicles compared to the control (Figure 3.2D, n = 4 – 12 dams/treatment group; $p \leq 0.05$).

Effect of DEHP exposure on F2 ovarian morphology

At PND 1, exposure to DEHP did not significantly affect the number of germ cells, or primordial, primary, and total follicles compared to the control (Figure 3.3A). In contrast, at PND 8, the 200 mg/kg/day dose of DEHP significantly decreased the numbers of primordial, primary, and total follicles compared to the control (Figure 3.3B, n = 5 – 7 dams/treatment group; $p \leq 0.05$), but the decrease in primary follicle type only trended towards significance (Figure 3.3B, n = 5 – 7 dams/treatment group; p = 0.062). At PND 21, the 200 mg/kg/day dose of DEHP significantly decreased the number of primordial, primary, and total follicles compared to the control (Figure 3.3C, n = 5 – 7 dams/treatment group; $p \leq 0.05$). Further, the 500 mg/kg/day dose of DEHP decreased the number of primordial follicles, but the decrease only trended towards significance (Figure 3.3C, n = 4 – 7 dams/treatment group; p = 0.09). Both the 20 μg/kg/day and the 750 mg/kg/day doses of DEHP significantly decreased the number of

antral follicles at PND 21 compared to the control (Figure 3.3C, n = 5 – 7 dams/treatment group; $p \leq 0.05$). At PND 60, both the 200 µg/kg/day and the 750 mg/kg/day doses of DEHP decreased the number of antral follicle numbers compared to the control (Figure 3.3D, n = 6 – 11dams/treatment group; $p \leq 0.05$). Further, the 20 µg/kg/day dose of DEHP decreased the total number of follicles, but the decrease only trended towards significance (Figure 3.3D, n = 8 – 11 dams/treatment group; $p = 0.075$).

At PND 1, the 200 µg/kg/day dose of DEHP decreased the percentage of germ cells and increased the percentage of primordial follicles types, but these changes only trended towards significance (Figure 3.4A, n = 3 – 4 dams/treatment group; $p = 0.077$ and $p = 0.077$, respectfully). At PND 8, exposure to both the 20 µg/kg/day and the 200 mg/kg/day doses of DEHP decreased the percentage of primordial follicles, but the decreases only trended towards significance (Figure 3.4B, n = 4 – 7 dams/treatment group; $p = 0.088$ and $p = 0.062$). Further, the 200 mg/kg/day dose of DEHP significantly increased the percentage of preantral follicles compared to controls (Figure 3.4B, n = 5 – 7 dams/treatment group; $p \leq 0.05$). At PND 21, the 200 mg/kg/day of DEHP decreased the percentage of primordial follicles compared to the control, but the decrease only trended towards significance (Figure 3.4C, n = 5 – 7 dams/treatment group; $p = 0.058$), whereas the 500 mg/kg/day dose of DEHP significantly decreased the percentage of primordial follicles compared to the control (Figure 3.4C, n = 6 – 7 dams/treatment group; $p \leq 0.05$). The 200 and 500 mg/kg/day doses of DEHP significantly increased the percentage of preantral follicles compared to the control, with the increase in the 500 mg/kg/day trending towards significance (Figure 3.4C, n = 4 – 7 dams/treatment group; $p \leq 0.05$ and $p = 0.086$, respectfully). The 20 µg/kg/day significantly decreased the percentage of antral follicles and the 200 mg/kg/day doses of DEHP significantly increased the percentage of

antral follicles compared to the control (Figure 3.4C, n = 4 – 7 dams/treatment group; $p \leq 0.05$). At PND 60, the 750 mg/kg/day dose of DEHP increased the percentage of primary follicles and decreased the percentage atretic follicles, but it only trended towards significance (Figure 3.4D, n = 5 – 11 dams/treatment group; p = 0.070 and p = 0.066, respectfully). Further, the 200 µg/kg/day dose of DEHP significantly decreased the percentage of preantral and antral follicles compared to controls, but the decrease in preantral follicles only trended towards significance (Figure 3.4D, n = 7 – 11 dams/treatment group; p = 0.094 and $p \leq 0.05$, respectfully).

Effect of DEHP exposure on F3 ovarian morphology

At PND 1, the 500 mg/kg/day dose of DEHP significantly decreased the number of germ cells and total number of oocytes compared to the control (Figure 3.5A, n = 5 – 7 dams/treatment group; $p \leq 0.05$). At PND 8, the 200 µg/kg/day and the 500 mg/kg/day doses of DEHP increased the number of primordial, preantral, and total follicles compared to the control (Figure 3.5B, n = 4 – 5 dams/treatment group; $p \leq 0.05$, except 500 mg/kg/day preantral p = 0.088). At PND 21, the 20 µg/kg/day dose of DEHP significantly decreased the number of primordial and total number of follicles compared to the control (Figure 3.5C, n = 4 – 6 dams/treatment group; $p \leq 0.05$). The 750 mg/kg/day dose of DEHP decreased the number of preantral follicles, but the decrease only trended towards significance (Figure 3.5C, n = 5 dams/treatment group; p = 0.068). At PND 60, exposure to DEHP did not significantly affect the numbers of primordial, primary, preantral, antral, and total follicles compared to the control (Figure 3.5D).

At PND 1, both the 500 mg/kg/day and the 750 mg/kg/day doses of DEHP significantly decreased the percentage of germ cells and increased the percentage of primordial follicle types compared to the control (Figure 3.6A, n = 5 – 7 dams/treatment group; $p \leq 0.05$). At PND 8, the

20 µg/kg/day dose of DEHP significantly increased the percentage of preantral follicles compared to the control (Figure 3.6B, n = 5 – 7 dams/treatment group; $p \leq 0.05$). At PNDs 21 and 60, DEHP exposure did not significantly affect the percentage of primordial, primary, preantral, antral, or atretic follicles compared to the control (Figures 3.6C and 3.6D).

Effect of DEHP on corpora lutea in all generations

At PND 60, exposure to DEHP did not significantly affect the number of corpora lutea in the F1 generation (Figure 3.7). Similarly, DEHP exposure did not significantly affect the number of corpora lutea in the F2 and F3 generations (Figure 3.7).

Effect of DEHP exposure on serum 17β-estradiol levels

At PND 8, the 20 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day doses of DEHP significantly increased serum 17β-estradiol levels compared to the control in the F1 generation (Figure 3.8A, n = 5 – 8 dams/treatment group; $p \leq 0.05$, except for 500 mg/kg/day $p = 0.057$). In contrast, the 750 mg/kg/day dose of DEHP decreased serum 17β-estradiol levels in the F2 generation and only trended towards significance (Figure 3.8A, n = 5 – 10 dams/treatment group; $p = 0.09$). DEHP exposure did not significantly affect serum 17β-estradiol levels in the F3 generation (Figure 3.8A).

At PND 21, exposure to DEHP did not significantly affect serum 17β-estradiol levels in the F1 generation. In contrast, exposure to DEHP decreased serum 17β-estradiol levels in the 20 µg/kg/day treatment group in the F2 generation, but the decrease only trended towards significance (Figure 3.8B, n = 7 – 9 dams/treatment group; $p = 0.074$). Exposure to DEHP did not significantly affect serum 17β-estradiol levels in the F3 generation (Figure 3.8B)

At PND 60, exposure to the 500 mg/kg/day dose of DEHP significantly increased serum 17β-estradiol levels compared to the control in the F1 generation (Figure 3.8C, n = 7 dams/treatment group; $p \leq 0.05$). In contrast, DEHP exposure did not significantly affect serum 17β-estradiol levels in the F2 and F3 generations (Figure 3.8C).

Effect of DEHP exposure on serum progesterone levels

At PND 21, exposure to 20 µg/kg/day and 500 mg/kg/day doses of DEHP significantly increased serum progesterone levels compared to the control in the F2 generation (Figure 3.8D, n = 5 – 8 dams/treatment group; $p \leq 0.05$ except 20 µg/kg/day $p = 0.057$). In contrast, DEHP exposure did not significantly affect serum progesterone levels in the F3 generation (Figure 3.8D).

At PND 60, serum progesterone levels were not significantly affected by DEHP exposure in the F1 generation (Figure 3.8E). In contrast, exposure to 200 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day dose of DEHP significantly decreased serum progesterone levels compared to the control in the F2 generation (Figure 3.8E, n = 3 – 10 dams/treatment group; $p \leq 0.05$ except 200 µg/kg/day $p = 0.091$). DEHP exposure did not significantly affect progesterone levels in the F3 generation (Figure 3.8E).

Effect of DEHP exposure on serum testosterone levels

At PND 60, exposure to 200 µg/kg/day of DEHP significantly decreased serum testosterone levels compared to the control in the F1 generation (Figure 3.8F, n = 4 – 7 dams/treatment group; $p \leq 0.05$). In contrast, DEHP exposure did not significantly affect serum testosterone levels in the F2 and F3 generations.

Effect of DEHP exposure on serum gonadotropin hormone levels

Exposure to DEHP did not significantly affect serum LH and serum FSH levels in the F1 generation. (Figures 3.9G and 3.9H). Similarly, DEHP exposure did not significantly affect serum LH and FSH levels in the F2 and F3 generations (Figures 3.9G and 3.9H).

3.5 Discussion

We have shown that prenatal exposure to DEHP during the second half of pregnancy disrupts ovarian function across generations through the maternal lineage in mice. Specifically, we provide evidence that DEHP exposure adversely affects body weights in the F2 generation, tissue weights in the F1 and F2 generations, ovarian morphology in the F1, F2, and F3 generations, and serum sex steroid hormone levels in the F1 and F2 generations. Further, to our knowledge, we are the first to show adverse transgenerational effects of prenatal DEHP exposure on ovarian morphology in detail.

Interestingly, the effects caused by DEHP exposure were not the same in each generation and many effects were non-monotonic in nature. This is likely because during prenatal exposure to DEHP, the F1, F2, and F3 generations received DEHP during different developmental windows. Specifically, the F1 generation was exposed to DEHP as the developing pup. The F2 generation was exposed to DEHP as the developing ovaries within the pup. The F3 generation was not directly exposed to DEHP. Further, DEHP is a known endocrine disrupting chemical (EDC), and characteristic of an EDC is that the adverse effects of a toxicant are not proportional to the dose and therefore, often do not follow a linear relationship [44]. Further, because DEHP is rapidly metabolized and excreted with a half-life between 2 – 8 hours [45], it is important to

note that the exposure window to DEHP is limited. Thus, it is unlikely that DEHP levels during gestation continued into the F1, F2, and F3 generations.

Our data showed that DEHP exposure decreased body weight in the F2 generation at PNDs 8 and 21, but it did not affect body weight in the F1 and F3 generations. This is in contrast to a previous study in which prenatal exposure to a phthalate mixture (200 µg/kg/day and 200 mg/kg/day) increased body weight in the F2 generation of female mice [46]. The reasons why our results differ may be because the phthalate mixture contained multiple phthalates in addition to DEHP and the different phthalates could have affected the body weight differently than DEHP alone. In addition, the dose of DEHP in the phthalate mixture was different than the doses we used in this study (approximately 41 µg and 41 mg/kg/day in the mixture vs. 20 µg – 750 mg/kg/day in this study) [46].

The mechanism by which DEHP exposure decreased body weight in our study is not known. However, phthalates such as DEHP have been shown to act on peroxisome proliferator-activated receptors (PPARs) and these receptors are important for the regulation of glucose homeostasis and in turn, body weight [47]. Therefore, it is possible that DEHP may have acted on PPARs to dysregulate body weight in our study. It is interesting to note that the decrease in body weight was observed only with the lowest dose of DEHP (20 µg/kg/day), suggesting that different doses of DEHP may act on the body differently. This possibility is supported by another study that showed that prenatal DEHP exposure caused different effects on body weight at different doses. Specifically, prenatal DEHP increased body weight at 200 µg/kg/day and decreased body weight at 500 mg/kg/day in male mice [13].

In addition, our data showed that prenatal DEHP exposure affected ovarian weight in the F1 and F2 generations. Specifically, in the F1 generation, the 20 µg/kg/day and 750 mg/kg/day

doses of DEHP increased ovarian weight and the 200 µg/kg/day dose decreased ovarian weight at PND 21. In the F2 generation, DEHP exposure at 20 µg/kg/day decreased ovarian weight. The reasons why DEHP causes ovarian weight changes are unknown. We speculate that DEHP exposure may alter the granulosa cell size, the granulosa cell number, or affect the interstitial cells, leading to alterations in ovarian weight. The DEHP-induced decrease in ovarian weight is similar to the DEHP-induced decrease in testes weight observed after prenatal exposure to 325 µL/L of DEHP in drinking water [48]. However, this level of DEHP in drinking water is equivalent to 30 – 35 mg/kg/day of DEHP and is much higher than the dose at which we observed a decrease in ovarian weight [48]. Our data differ from another study showing that prenatal DEHP exposure (0.015 – 405 mg/kg/day) from gestation day 6 to lactation day 21 did not affect ovarian weight [49]. The difference in results between our study and this study may be due to the different windows of exposure (during second half of gestation vs. through lactation), different doses of DEHP, different animal models (mice vs. rats), and different methods of exposure (gavage vs oral).

Prenatal DEHP exposure also increased uterine weight at PND 21 in the F1 generation, but it did not significantly affect uterine weight at any time points in the F2 or F3 generations. An increase in uterine weight may correlate with an increase in 17β-estradiol production. However, our data showed that at PND 21, DEHP exposure did not increase serum 17β-estradiol levels compared to controls. Thus, the DEHP-induced increase in uterine weight in the F1 generation at PND 21 may be due to developmental effects independent of 17β-estradiol levels. In contrast to our data, a study reported that *in utero* and lactational exposure to DEHP (0.015 – 405 mg/kg/day) did not alter uterine weight in the F1 generation of rats [49], suggesting the existence of species differences in the impact of prenatal DEHP exposure on uterine weight.

In our study, we counted follicle numbers and calculated the percentage of follicles at each stage of development. By examining the follicle numbers, we were able to quantify DEHP-induced changes in the number of available primordial follicles or the growth of follicles to more mature stages. By calculating the percentage of follicle types, we were able to quantify shifts in follicle pools, which may indicate either accelerated or inhibited folliculogenesis. Our data showed that the DEHP treatment disrupted ovarian follicle numbers in all three generations. In the F1 and F2 generations, exposure to DEHP decreased follicle numbers. However, in the F3 generation, DEHP exposure both decreased and increased follicle numbers, depending on the time point. Previous studies have reported that DEHP exposure during adulthood decreases the primordial follicle pool [34, 50]. These findings indicate that both prenatal and adult exposure to DEHP impact follicle numbers. The impact of prenatal exposure to DEHP is especially concerning because the DEHP-induced decrease in follicle numbers occurs across generations. It is possible that DEHP-induced losses in the finite number of primordial follicles may cause primordial follicle ovarian insufficiency, leading to declining fertility with age, and an early age of reproductive senescence. Loss of follicles was also observed in another study. Specifically, prenatal exposure to a plasticizer mixture including DEHP (750 mg/kg/day) reduced the number of primordial follicles in the F1 and F3 generations of female rats [37]. Taken together, these results suggest that prenatal DEHP exposure may lead to primary ovarian insufficiency, which is the premature loss of ovarian follicles caused by either atresia of follicular destruction [51]. Thus, future studies should examine the impact of DEHP-induced follicle losses on fertility.

Our data showed that DEHP exposure decreased folliculogenesis in adult ovaries in the F1 generation, disrupted folliculogenesis in adult ovaries in the F2 generation, and accelerated the transition of germ cells to primordial follicles in the neonatal ovary in the F3 generation. The

mechanism by which DEHP exposure accelerates folliculogenesis, however, is not completely known. Our laboratory has previously demonstrated that adult exposure to DEHP (20 and 200 μg/kg/day and 20 and 750 mg/kg/day) for ten days accelerated primordial follicle recruitment in mouse ovaries, and that this acceleration was mediated through the phosphatidylinositol 3-kinase (PI3K) signaling pathway [34]. The PI3K pathway is a critical regulator of folliculogenesis, specifically for primordial follicle activation, survival, and quiescence [34]. Thus, it is possible that prenatal exposure to DEHP activated the PI3K signaling pathway in our study. In addition, another study showed that impaired folliculogenesis correlated with reduced progesterone levels during diestrus and thus, DEHP may impair folliculogenesis by interfering with progesterone levels [52]. This possibility is supported by our data indicating that DEHP exposure both reduced serum progesterone levels in the F2 generation and accelerated folliculogenesis. Further, studies show DEHP exposure (10 and 100 μM) *in vitro* impairs primordial follicle assembly by increasing the mRNA expression levels of pro-apoptotic gene *Bax* in oocytes [53]. Given that *Bax* plays an important role in follicle growth, it is possible that the mechanism by which DEHP impacts folliculogenesis involves *Bax* [54].

In addition, our data showed that prenatal exposure to DEHP decreased the percentage of atretic follicles in the F1 generation. Although the reasons for the DEHP-induced reduction in atresia is unknown, a previous study showed that a mixture of phthalates containing DEHP caused the antral follicles to become arrested in the G1 state of the cell cycle [55]. At this stage, the cells are relatively resistance to apoptosis [56]. Therefore, the DEHP-induced reduction in the percentage of atretic follicles may be due to an ability of DEHP to arrest granulosa cells in the antral follicles in the G1 stage.

Our data showed that prenatal DEHP exposure did not affect the number of corpora lutea in the ovaries in any generation. This is in contrast to previous studies that have shown that DEHP exposure at much higher doses (3,000 mg/kg) decreased the number of new corpora lutea in adult rats [57] and that DEHP exposure (125, 250, and 500 mg/kg/day) reduced the number of corpora lutea in pregnant mice [58]. Interestingly, one study showed that DEHP exposure (25 and 50 mg/kg/) reduced the size of corpora lutea, but increased plasma progesterone levels in sheep [59]. The reasons for differences in study results likely stem from differences in exposure windows (pregnancy vs. adulthood), species (mice vs. ewes), and timing of evaluation (pregnant dams vs. offspring).

Our data showed that DEHP exposure disrupted sex steroid hormone levels in the F1 and F2 generations. Specifically, in the F1 generation, prenatal DEHP exposure increased serum 17β-estradiol levels at PNDs 8 and 60. At PND 8, the increase in serum 17β-estradiol levels occurred at a time in which the ovary contained no antral follicles and because antral follicles are the primary produces of 17β-estradiol. These data suggest that the DEHP-induced increase in serum 17β-estradiol at PND 8 is not due to a significant increase in antral follicle numbers and that DEHP affects follicle numbers and sex steroid hormone levels via different mechanisms. One study has shown that disruption of FSH production can signal to the immature ovary and promote the synthesis of 17β-estradiol without stimulating follicular growth [60]. Therefore, it is possible that DEHP may promote FSH production and elevate serum 17β-estradiol levels. At PND 60, the ovary contains many antral follicles, but it is unlikely that the DEHP-induced increase in 17β-estradiol levels at this time point is due to a change in antral follicle numbers because we did not observe any effects of DEHP on antral follicle numbers or atresia at PND 60. Instead, it is possible that DEHP exposure may dysregulate the biosynthesis of 17β-estradiol in

the ovary. Previous studies have shown that *in vitro* DEHP exposure (1 – 100 µg/mL) decreases 17β-estradiol production [33] and decreases mRNA expression of aromatase, a key steroidogenic enzyme necessary for the conversion of cholesterol to 17β-estradiol in antral follicles [61]. Therefore, DEHP exposure *in vivo* may affect the levels of steroidogenic enzymes, increasing the levels of serum 17β-estradiol.

Our data showed that in the F2 generation, exposure to DEHP increased serum progesterone levels at PND 21 and decreased serum progesterone levels at PND 60. It is interesting that in the F2 generation, exposure to DEHP increased serum progesterone levels even though corpora lutea are not present at PND 21. One study has shown that FSH stimulation can promote progesterone synthesis and output from granulosa cells without luteinization by upregulating enzymatic activity of 3B-hydroxysteroid dehydrogenase (3BHSD) [62]. Although we did not measure FSH or 3BHSD levels in the F2 generation at PND 21, it is possible that DEHP exposure stimulated FSH production leading to elevated progesterone levels. At PND 60, serum progesterone levels were decreased in DEHP treated mice. Similarly, previous studies showed that *in vitro* DEHP exposure (1 – 100 µg/mL) decreased progesterone levels in antral follicles [33]. Further, DEHP exposure throughout lactation (1 – 100 mg/kg/day) decreased serum progesterone levels in adult female rats [63]. In addition, DEHP exposure has increased plasma progesterone levels by metabolic clearance and not by the level of secretion from the corpora lutea in sheep [58]. Further, DEHP exposure is associated with reduced hepatic estrogen metabolism and may contribute to the altered hormone levels observed in our study [64]. Therefore, hormone synthesis and clearance may contribute to the altered hormone levels observed in this study and should be further explored in future studies.

It is likely that DEHP exposure affects folliculogenesis and steroidogenesis through separate mechanisms. Our data show that DEHP exposure interferes with follicle numbers, but not sex steroid hormone levels or vice versa at corresponding time points. One possible mechanism that may explain the change in sex steroid hormone levels may be a disruption of FSH and LH action either directly on the follicles or through the hypothalamus-pituitary-ovary axis. Further, an alteration of the steroidogenic enzyme expression or activity in the follicles may also contribute to the altered hormone levels as demonstrated by a previous study [65]. A different mechanism to explain the change in follicle numbers, but not sex steroid hormone levels may be through an oxidative stress pathway. A previous study focusing on DEHP exposure in antral follicles has shown that DEHP (10 μg/mL) significantly increases reactive oxygen species levels and decreases antioxidant enzymes to inhibit antral follicle growth [66].

Collectively, these data provide evidence that prenatal exposure to DEHP during the second half of gestation causes body weight and organ weight changes, dysregulates serum sex steroid hormone levels, and causes adverse transgenerational changes in ovarian morphology. However, further work is needed to elucidate the mechanisms underlying the effects of DEHP on folliculogenesis and steroidogenesis across generations. The mechanisms underlying the direct effects of DEHP on the F1 and F2 generations may be very different compared to the effects of DEHP on the F3 generation. In the F1 and F2 generations, it is possible that the mechanisms involve direct effects of DEHP on peroxisome proliferator-activated receptor alpha, proliferator-activated receptor gamma, or estrogen receptors [67-69]. In the F3 generations, however, it is possible that the mechanism involves epigenetic modifications to the DNA, such as DNA methylation and histone modifications [18, 37, 70-72]. Normal folliculogenesis and

steroidogenesis are required for normal fertility. Therefore, future studies should also examine the fertility of mice prenatally exposed to DEHP in the F1, F2, and F3 generations.

3.6 Tables, Figures, and Legends

Table 3.1 Effects of prenatal exposure to DEHP on body weights, measured in grams, in the F1, F2, and F2 generations of female mice.

			GENERATION		
TIME POINT	Treatment Group	Sample Size	F1	F2	F3
PND 1	Control	4 - 17	1.792 ± 0.039	1.942 ± 0.142	1.851 ± 0.046
	DEHP 20 µg/kg/day	3 - 10	1.853 ± 0.065	1.786 ± 0.113	1.751 ± 0.048
	DEHP 200 µg/kg/day	3 - 10	1.886 ± 0.072	1.697 ± 0.124	1.751 ± 0.028
	DEHP 200 mg/kg/day	9	1.800 ± 0.062	no data	no data
	DEHP 500 mg/kg/day	3 - 10	1.861 ± 0.029	1.728 ± 0.054	1.790 ± 0.117
	DEHP 750 mg/kg/day	5 - 10	1.778 ± 0.033	no data	1.879 ± 0.099
PND 8	Control	15 - 20	6.300 ± 0.217	4.818 ± 0.115	5.116 ± 0.312
	DEHP 20 µg/kg/day	5- 9	6.200 ± 0.321	**4.238 ± 0.195 ***	5.038 ± 0.210
	DEHP 200 µg/kg/day	3 - 10	6.142 ± 0.345	5.027 ± 0.209	4.839 ± 0.145
	DEHP 200 mg/kg/day	8 - 9	5.580 ± 0.268	4.382 ± 0.191	no data
	DEHP 500 mg/kg/day	4 - 10	5.936 ± 0.192	5.125 ± 0.149	4.789 ± 0.363
	DEHP 750 mg/kg/day	5 - 7	5.740 ± 0.230	4.954 ± 0.218	5.531 ± 0.250
PND 21	Control	8 - 16	15.33 ± 0.414	12.38 ± 0.444	12.43 ± 0.758
	DEHP 20 µg/kg/day	4 - 7	14.00 ± 1.408	**8.84 ± 0.703 ***	11.78 ± 0.521
	DEHP 200 µg/kg/day	3 - 8	14.68 ± 0.757	12.43 ± 0.618	11.81 ± 0.876
	DEHP 200 mg/kg/day	6 - 8	14.81 ± 0.814	11.50 ± 0.440	no data
	DEHP 500 mg/kg/day	3 - 5	13.94 ± 0.496	15.40 ± 1.567	13.02 ± 0.766
	DEHP 750 mg/kg/day	5 - 6	14.75 ± 0.618	12.58 ± 1.363	13.60 ± 0.726

Table 3.1 (cont.)

			GENERATION		
TIME POINT	Treatment Group	Sample Size	F1	F2	F3
PND 60	Control	7 - 24	29.66 ± 0.527	27.92 ± 1.056	29.01 ± 0.872
	DEHP 20 µg/kg/day	6 - 8	29.08 ± 1.177	26.99 ± 0.726	26.75 ± 0.492
	DEHP 200 µg/kg/day	4 - 7	28.92 ± 1.517	27.57 ± 0.961	28.29 ± 1.331
	DEHP 200 mg/kg/day	9	30.30 ± 0.513	no data	no data
	DEHP 500 mg/kg/day	3 - 11	27.93 ± 0.938	29.72 ± 2.752	29.70 ± 1.688
	DEHP 750 mg/kg/day	4 - 6	28.16 ± 0.189	28.96 ± 1.671	29.25 ± 1.458
3 MONTH	Control	8 - 14	32.45 ± 1.105	29.47 ± 1.308	32.15 ± 0.742
	DEHP 20 µg/kg/day	7 - 8	31.66 ± 1.385	29.30 ± 0.510	29.59 ± 0.719
	DEHP 200 µg/kg/day	4 - 10	31.35 ± 0.684	29.15 ± 0.519	30.05 ± 0.484
	DEHP 200 mg/kg/day	0	no data	no data	no data
	DEHP 500 mg/kg/day	3 - 13	30.96 ± 1.030	30.88 ± 0.392	31.25 ± 1.273
	DEHP 750 mg/kg/day	6 - 7	33.55 ± 0.988	28.53 ± 0.821	31.23 ± 1.827
6 MONTH	Control	7 - 14	39.63 ± 1.505	39.97 ± 1.379	41.48 ± 1.461
	DEHP 20 µg/kg/day	6 - 8	39.94 ± 1.757	36.71 ± 1.309	36.92 ± 0.414
	DEHP 200 µg/kg/day	3 - 10	37.76 ± 1.302	39.70 ± 1.841	38.07 ± 1.784
	DEHP 200 mg/kg/day	0	no data	no data	no data
	DEHP 500 mg/kg/day	3 - 13	37.18 ± 1.169	40.48 ± 0.273	40.33 ± 2.497
	DEHP 750 mg/kg/day	6 - 7	40.31 ± 1.344	37.31 ± 0.757	39.01 ± 1.306

Table 3.1 (cont.)

			GENERATION		
TIME POINT	Treatment Group	Sample Size	F1	F2	F3
9 MONTH	Control	7 - 14	41.21 ± 1.377	42.28 ± 2.319	46.10 ± 2.120
	DEHP 20 µg/kg/day	6 - 8	41.19 ± 1.824	39.83 ± 2.255	41.70 ± 0.817
	DEHP 200 µg/kg/day	3 - 10	40.61 ± 1.524	42.54 ± 2.205	45.27 ± 2.739
	DEHP 200 mg/kg/day	0	no data	no data	no data
	DEHP 500 mg/kg/day	3 - 13	39.26 ± 1.201	44.93 ± 1.282	47.90 ± 3.731
	DEHP 750 mg/kg/day	6 - 7	43.61 ± 1.691	40.21 ± 1.316	43.73 ± 2.312

Note: Table represent means ± standard error of the mean from 3 – 24 dams per treatment group.

*$p \leq 0.05$ (significant difference compared to the control)

Table 3.2 Effects of prenatal exposure to DEHP on ovarian weights, measured in milligrams, in the F1, F2, and F3 generations of female mice.

			GENERATION		
TIME POINT	Treatment Group	Sample Size	F1	F2	F3
PND 21	Control	8 - 15	6.7 ± 0.5	5.0 ± 0.4	4.6 ± 0.5
	DEHP 20 µg/kg/day	3 - 7	**3.9 ± 0.3 ***	4.2 ± 0.4	4.1 ± 0.2
	DEHP 200 µg/kg/day	3 - 8	**34.3 ± 5.9 ***	5.1 ± 0.5	5.1 ± 0.3
	DEHP 200 mg/kg/day	6 - 8	32.8 ± 16.4 ^	5.3 ± 0.9	no data
	DEHP 500 mg/kg/day	3 - 5	6.1 ± 1.0	6.2 ± 0.8	4.5 ± 0.3
	DEHP 750 mg/kg/day	5 - 6	**5.1 ± 0.2 ***	6.8 ± 0.7	4.6 ± 0.4
PND 60	Control	7 - 24	15.4 ± 0.8	20.7 ± 1.8	16.9 ± 1.6
	DEHP 20 µg/kg/day	6 - 7	14.0 ± 1.2	16.0 ± 1.2 ^	14.2 ± 1.4
	DEHP 200 µg/kg/day	4 - 7	14.1 ± 1.7	**15.0 ± 0.8 ***	16.0 ± 1.3
	DEHP 200 mg/kg/day	9	14.2 ± 1.4	no data	no data
	DEHP 500 mg/kg/day	3 - 11	14.6 ± 1.0	17.6 ± 2.0	17.3 ± 1.4
	DEHP 750 mg/kg/day	5 - 6	14.5 ± 0.4	19.9 ± 1.6	15.3 ± 1.3

Note: Ovarian weights were not collected in the F1, F2, or F3 generations at PND 1 or PND 8. Table represent means ± standard error of the mean from 3 – 24 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); $0.05 > {}^{\wedge}p \geq 0.076$ (borderline significant difference compared to the control).

Table 3.3 Effects of prenatal exposure to DEHP on uterine weights, measured in milligrams, in the F1, F2, and F3 generations of female mice.

			GENERATION		
TIME POINT	Treatment Group	Sample Size	F1	F2	F3
PND 1	Control	7	no data	no data	1.0 ± 0.1
	DEHP 20 µg/kg/day	7			1.1 ± 0.1
	DEHP 200 µg/kg/day	3			1.1 ± 0.1
	DEHP 200 mg/kg/day	0			no data
	DEHP 500 mg/kg/day	3			1.1 ± 0.2
	DEHP 750 mg/kg/day	5			0.9 ± 0.2
PND 8	Control	8 - 15	56.1 ± 2.8	3.6 ± 0.2	4.4 ± 0.2
	DEHP 20 µg/kg/day	5 - 7	49.3 ± 4.8	3.5 ± 0.2	4.1 ± 0.3
	DEHP 200 µg/kg/day	3 - 8	56.2 ± 4.9	4.0 ± 0.2	4.6 ± 0.5
	DEHP 200 mg/kg/day	7 -9	66.8 ± 3.4	3.1 ± 0.1 ^	no data
	DEHP 500 mg/kg/day	3 - 5	61.3 ± 4.8	**5.1 ± 0.8 ***	4.0 ± 0.4
	DEHP 750 mg/kg/day	5 - 7	55.2 ± 4.8	4.1 ± 0.3	4.5 ± 0.2
PND 21	Control	8 - 16	20.6 ± 1.8	16.4 ± 1.4	15.3 ± 1.0
	DEHP 20 µg/kg/day	4 - 7	15.3 ± 2.5	**9.3 ± 0.7 ***	12.5 ± 1.2
	DEHP 200 µg/kg/day	3 - 7	**206.0 ± 26.9 ***	14.2 ± 0.9	15.1 ± 1.3
	DEHP 200 mg/kg/day	6 - 8	50.2 ± 17.1	16.4 ± 2.2	no data
	DEHP 500 mg/kg/day	3 - 5	18.7 ± 2.5	21.2 ± 5.4	17.7 ± 1.6
	DEHP 750 mg/kg/day	4 - 6	23.3 ± 2.5	13.4 ± 1.3	17.5 ± 0.7

Table 3.3 (cont.)

			GENERATION		
TIME POINT	Treatment Group	Sample Size	F1	F2	F3
PND 60	Control	6 - 7	111.2 ± 5.6	128.5 ± 15.8	106.0 ± 7.3
	DEHP 20 µg/kg/day	4 - 7	123.2 ± 5.7	107.3 ± 7.5	116.2 ± 17.5
	DEHP 200 µg/kg/day	4	131.7 ± 16.7	153.0 ± 19.6	95.8 ± 3.8
	DEHP 200 mg/kg/day	0	no data	no data	no data
	DEHP 500 mg/kg/day	2 - 7	110.3 ± 9.2	103.3 ± 15.2	145.1 ± 33.7
	DEHP 750 mg/kg/day	4 - 6	no data	118.0 ± 28.3	99.2 ± 2.8

Note: Uterine weights were not collected in the F1 and F2 generations at PND 1. Table represent means ± standard error of the mean from 2 – 16 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); $0.05 > \text{^}p \geq 0.084$ (borderline significant difference compared to the control).

Table 3.4 Effects of prenatal exposure to DEHP on liver weights, measured in grams, in the F1, F2, and F3 generations of female mice.

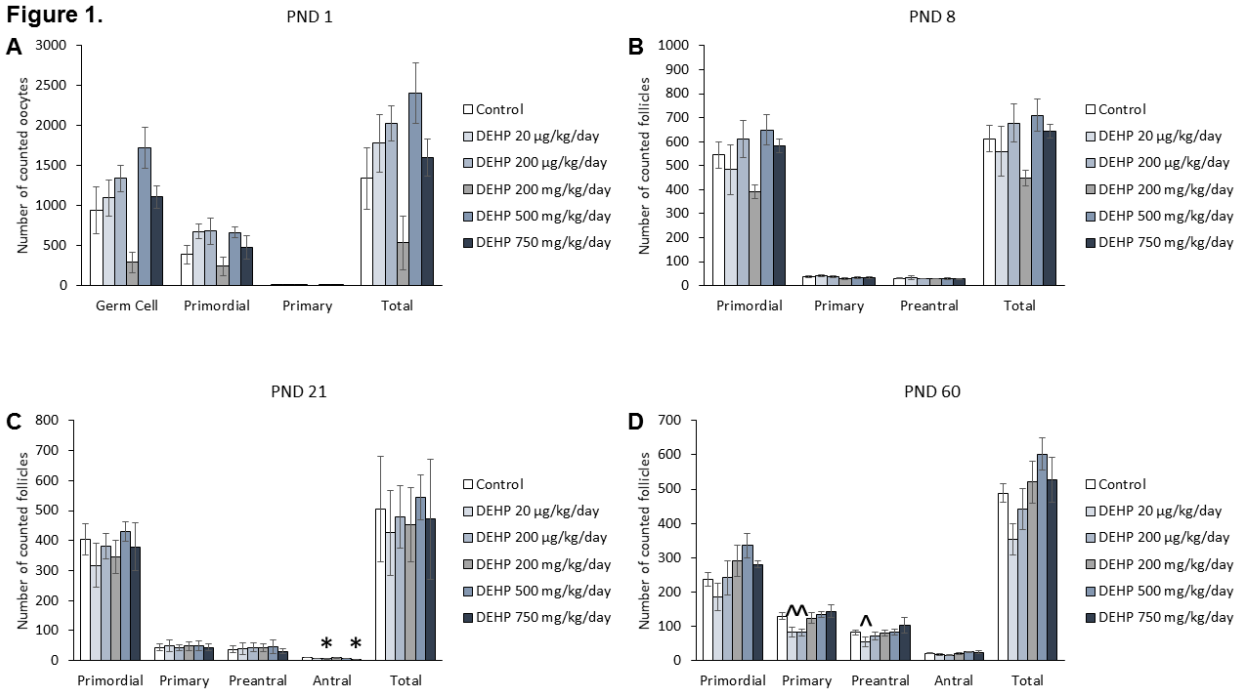
			GENERATION		
TIME POINT	Treatment Group	Sample Size	F1	F2	F3
PND 1	Control	5 - 17	0.0731 ± 0.0022	0.0745 ± 0.0053	0.0721 ± 0.0027
	DEHP 20 µg/kg/day	3 - 7	0.0720 ± 0.0037	0.0710 ± 0.0045	0.0728 ± 0.0029
	DEHP 200 µg/kg/day	3 - 6	0.0733 ± 0.0033	0.0679 ± 0.0032	0.0748 ± 0.0037
	DEHP 200 mg/kg/day	9	0.0700 ± 0.0041	no data	no data
	DEHP 500 mg/kg/day	3 - 9	**0.0840 ± 0.0024 ***	0.0721 ± 0.0027	0.0723 ± 0.0037
	DEHP 750 mg/kg/day	5	0.0760 ± 0.0024	no data	0.0766 ± 0.0049
PND 8	Control	8 - 17	0.1950 ± 0.0086	0.1234 ± 0.0088	0.1621 ± 0.0139
	DEHP 20 µg/kg/day	5 - 9	0.1920 ± 0.0139	0.1207 ± 0.0076	0.1481 ± 0.0093
	DEHP 200 µg/kg/day	3 - 10	0.1840 ± 0.0147	0.1491 ± 0.0097	0.1461 ± 0.0071
	DEHP 200 mg/kg/day	8 - 9	**0.1563 ± 0.0091 ***	0.1137 ± 0.0053	no data
	DEHP 500 mg/kg/day	4 - 10	0.1780 ± 0.0153	**0.1577 ± 0.0085 ***	0.1475 ± 0.0077
	DEHP 750 mg/kg/day	5 - 7	0.1700 ± 0.0115	0.1434 ± 0.0085	0.1776 ± 0.0099
PND 21	Control	8 - 16	0.8154 ± 0.0256	0.6911 ± 0.0324	0.6654 ± 0.0505
	DEHP 20 µg/kg/day	4 - 7	0.7607 ± 0.0599	**0.4541 ± 0.0445 ***	0.6280 ± 0.0327
	DEHP 200 µg/kg/day	3 - 8	0.8220 ± 0.0496	0.6926 ± 0.0402	0.6305 ± 0.0708
	DEHP 200 mg/kg/day	6 - 8	0.7438 ± 0.0385	0.5865 ± 0.0246	no data
	DEHP 500 mg/kg/day	3 - 5	0.7565 ± 0.0206	**0.9616 ± 0.1299 ***	0.6928 ± 0.0413
	DEHP 750 mg/kg/day	5 - 6	0.8527 ± 0.0506	0.6968 ± 0.1106	0.7607 ± 0.0441

Table 3.4 (cont.)

			GENERATION		
TIME POINT	Treatment Group	Sample Size	F1	F2	F3
PND 60	Control	7 - 23	1.6533 ± 0.0384	1.6236 ± 0.0940	1.7297 ± 0.0641
	DEHP 20 µg/kg/day	6 - 8	1.6687 ± 0.0630	1.5593 ± 0.0425	1.5765 ± 0.0489
	DEHP 200 µg/kg/day	4 - 7	1.5630 ± 0.1100	1.5649 ± 0.0901	1.7190 ± 0.1990
	DEHP 200 mg/kg/day	8	1.5885 ± 0.0358	no data	no data
	DEHP 500 mg/kg/day	3 - 11	1.6264 ± 0.0609	1.7531 ± 0.1860	1.7792 ± 0.1131
	DEHP 750 mg/kg/day	5 - 6	1.5357 ± 0.0761	1.6317 ± 0.1257	1.7854 ± 0.1110

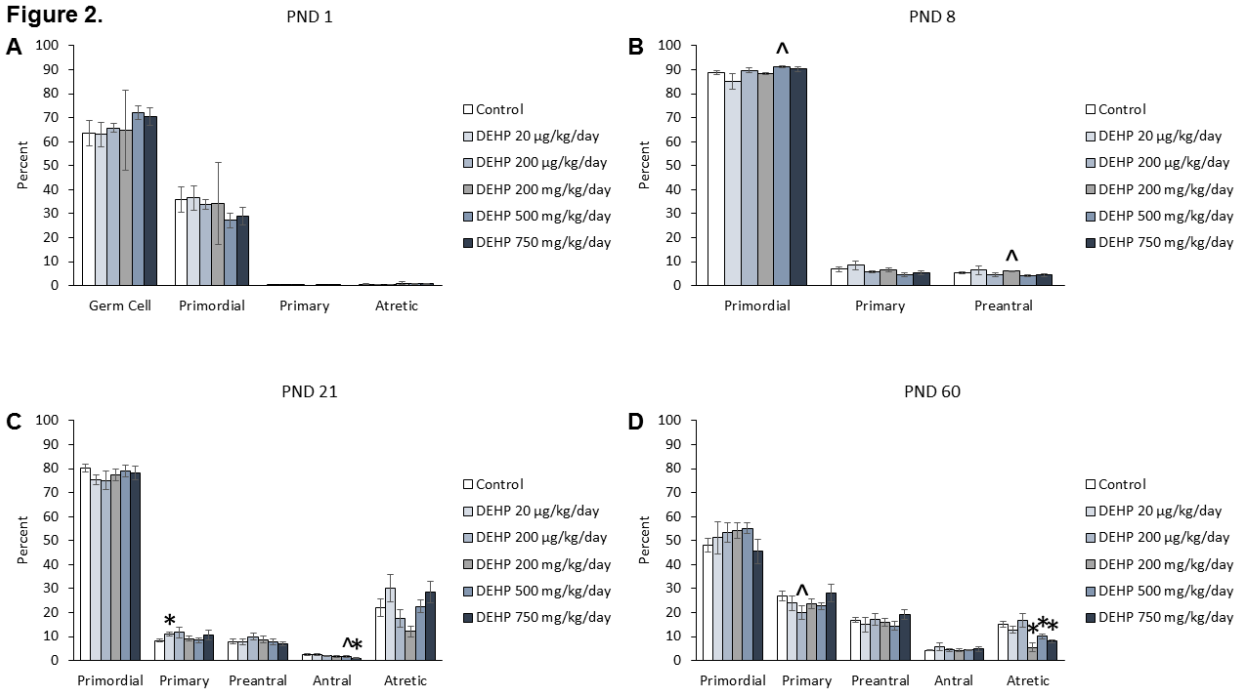
Note: Tables represent means ± standard error of the mean from 3 – 23 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control).

Figure 3.1 The effects of prenatal DEHP exposure on ovarian follicle numbers in the F1 generation



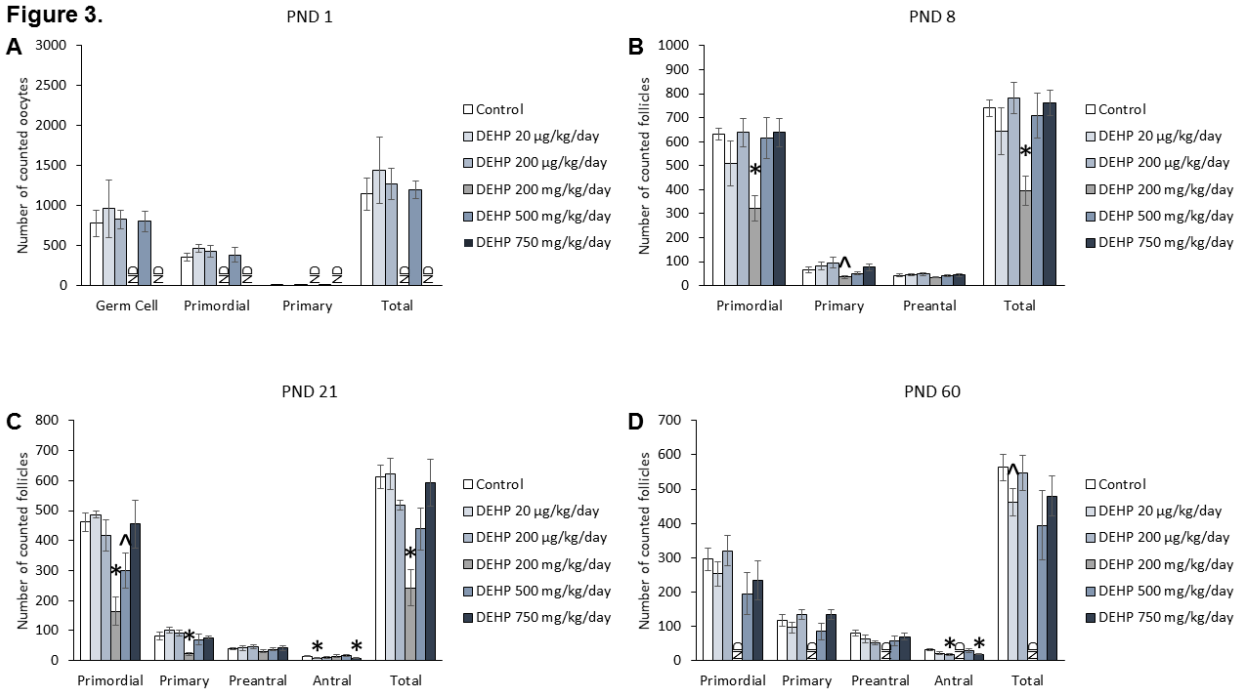
The effects of prenatal exposure to DEHP on ovarian follicle numbers in the F1 generation at PND 1 (A), PND 8 (B), PND 21 (C), and PND 60 (D). Graphs represent means ± standard error of the mean from 2 – 14 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); $0.05 > \text{^}p \geq 0.084$ (borderline significant difference compared to the control).

Figure 3.2 The effects of prenatal DEHP exposure on ovarian follicle percentages in the F1 generation



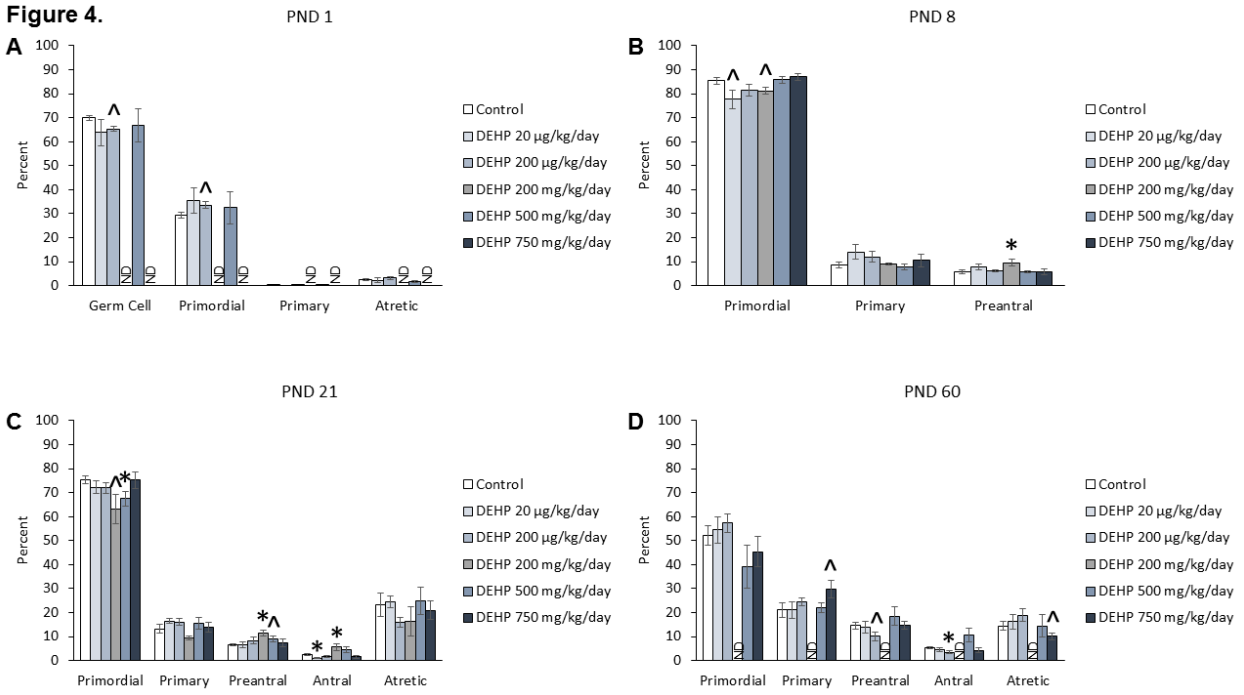
The effects of prenatal exposure to DEHP on ovarian follicle percentages in the F1 generation at PND 1 (A), PND 8 (B), PND 21 (C), and PND 60 (D). Graphs represent means ± standard error of the means from 2 – 14 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); $0.05 > \text{^}p \geq 0.085$ (borderline significant difference compared to the control).

Figure 3.3 The effects of prenatal DEHP exposure on ovarian follicle numbers in the F2 generation



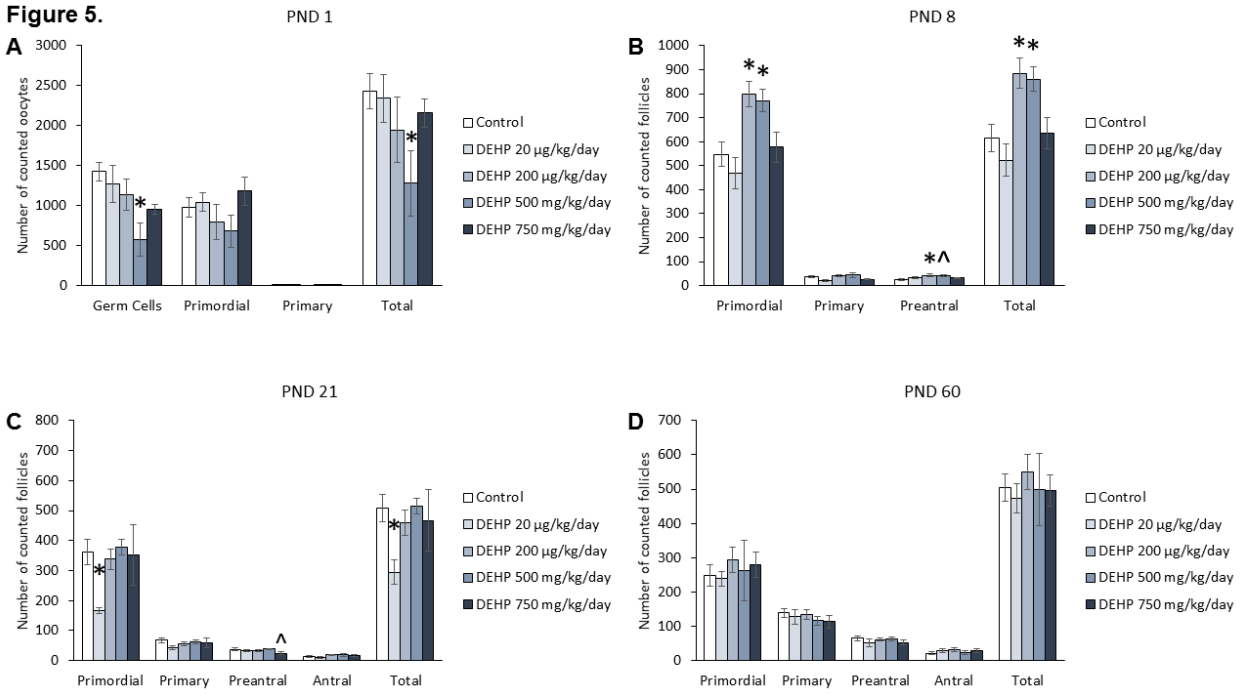
The effects of prenatal exposure to DEHP on ovarian follicle numbers in the F2 generation at PND 1 (A), PND 8 (B), PND 21 (C), and PND 60 (D). Graphs represent means ± standard error of the mean from 3 – 11 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); $0.05 > ^{}p \geq 0.09$ (borderline significant difference compared to the control). ND = no data

Figure 3.4 The effects of prenatal DEHP exposure on ovarian follicle percentages in the F2 generation



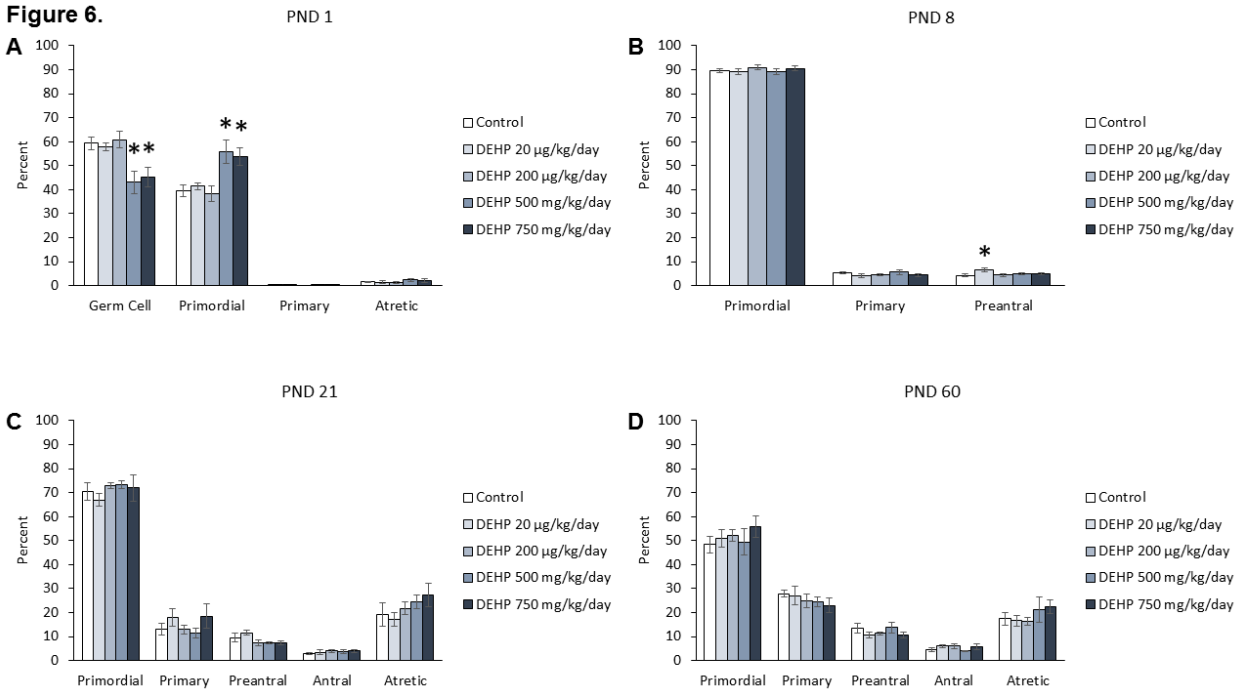
The effects of prenatal exposure to DEHP on ovarian follicle percentages in the F2 generation at PND 1 (A), PND 8 (B), PND 21 (C), and PND 60 (D). Graphs represent means ± standard error of the means from 3 – 11 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); $0.05 > \text{^}p \geq 0.094$ (borderline significant difference compared to the control). ND = no data

Figure 3.5 The effects of prenatal DEHP exposure on ovarian follicle numbers in the F3 generation



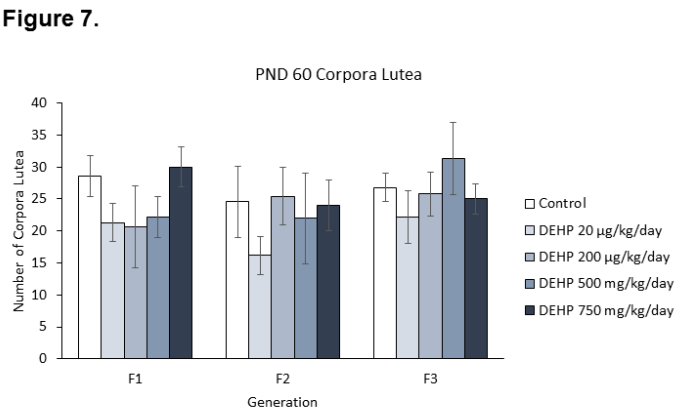
The effects of prenatal exposure to DEHP on ovarian follicle numbers in the F3 generation at PND 1 (A), PND 8 (B), PND 21 (C), and PND 60 (D). Graphs represent means ± standard error of the mean from 4 – 7 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); $0.05 >$ ^$p \geq 0.088$ (borderline significant difference compared to the control).

Figure 3.6 The effects of prenatal DEHP exposure on ovarian follicle percentages in the F3 generation



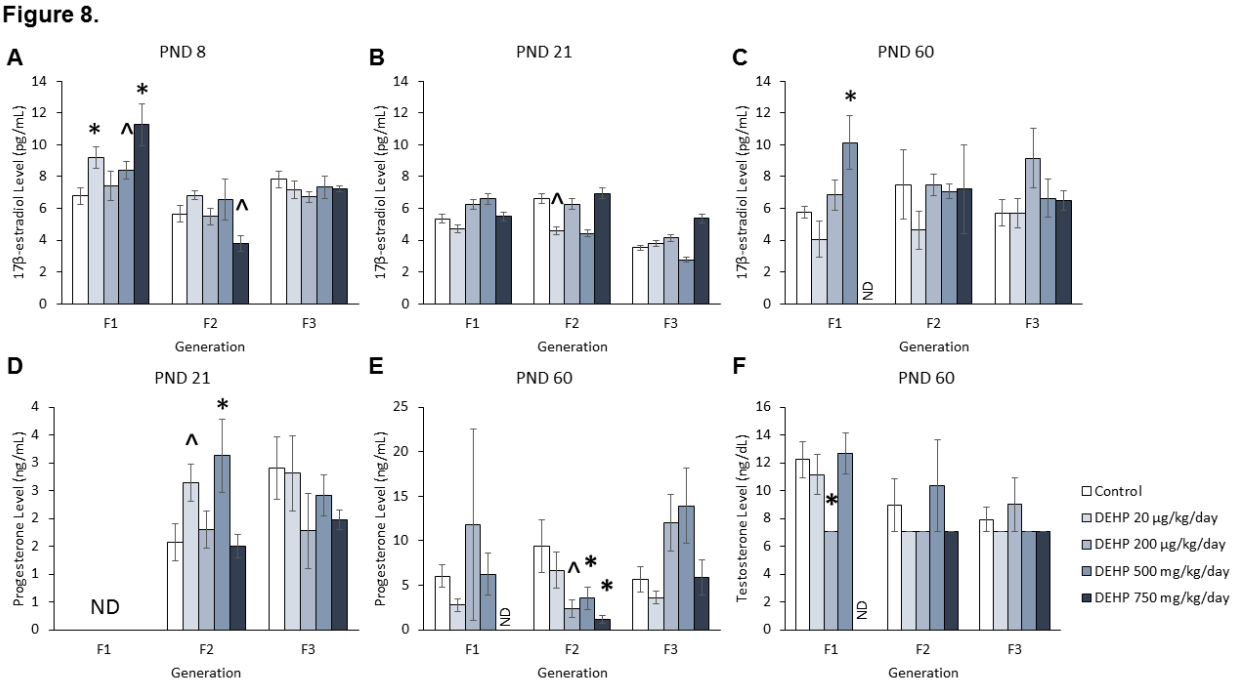
The effects of prenatal exposure to DEHP on ovarian follicle percentages in the F3 generation at PND 1 (A), PND 8 (B), PND 21 (C), and PND 60 (D). Graphs represent means ± standard error of the means from 4 – 7 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control).

Figure 3.7 The effects of prenatal DEHP exposure on corpora lutea in the F1 – F3 generations



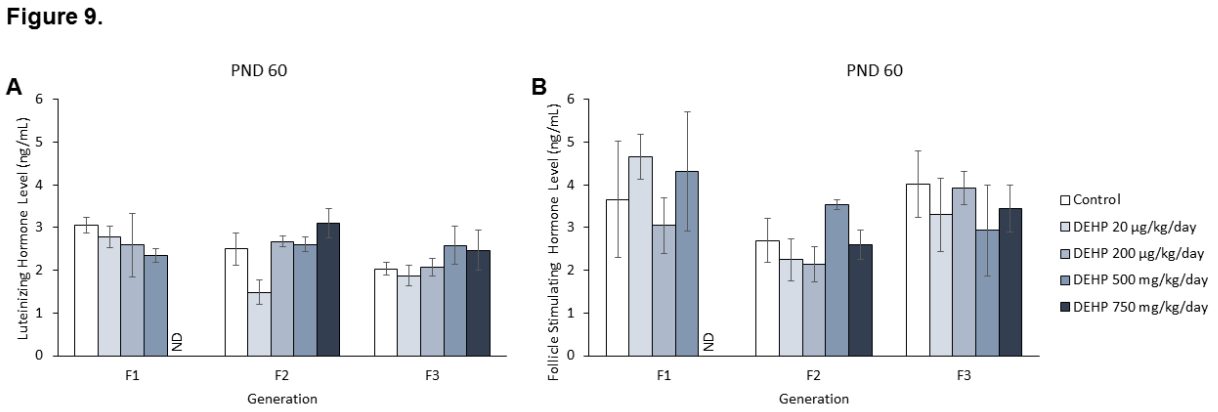
The effects of prenatal exposure to DEHP on corpora lutea numbers at PND 60. Graph represents mean ± standard error of the means from 4 – 12 dams per treatment group.

Figure 3.8 The effects of prenatal DEHP exposure on serum sex steroid hormone levels in F1 – F3 generations



The effects of prenatal exposure to DEHP on serum sex steroid hormone levels in the F1, F2, and F3 generations. Serum 17β-estradiol levels are shown at PND 8 (A), PND 21 (B) and PND 60 (C) for the F1, F2, and F3 generations. Serum progesterone levels are shown at PND 21 (D) and PND 60 (E) for the F1, F2, and F3 generations. Serum testosterone levels are shown at PND 60 (F) for the F1, F2, and F3 generations. The serum testosterone levels were at the limit of detection and therefore do not have error bars. Graphs represent means ± standard error of the mean from 2 – 8 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); $0.05 >$ ^$p \geq 0.091$ (borderline significant difference compared to the control); ND = no data

Figure 3.9 The effects of prenatal DEHP exposure on serum gonadotropin hormone levels in the F1 – F3 generations



The effects of prenatal exposure to DEHP on serum gonadotropin hormone levels in the F1, F2, and F3 generations at PND 60. Serum luteinizing hormone levels are shown in panel (A) and serum follicle stimulating hormone levels are shown in panel (B). Graphs represent means ± standard error of the mean from 2 – 10 dams per treatment group. ND = no data

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CHAPTER 4

Di(2-ethylhexyl) Phthalate Exposure During Prenatal Development Causes Adverse Transgenerational Effects on Female Fertility in Mice[2]

4.1 Abstract

Di(2-ethylhexyl) phthalate (DEHP) is a ubiquitous environmental toxicant and endocrine disrupting chemical, but little is known about its effects on female reproduction. Thus, we tested the hypothesis that prenatal exposure to DEHP accelerates the onset of puberty, disrupts estrous cyclicity, disrupts birth outcomes, and reduces fertility in the F1, F2, and F3 generations of female mice. Pregnant CD-1 mice were orally dosed with corn oil (vehicle control) or DEHP (20 and 200 µg/kg/day and 500 and 750 mg/kg/day) from gestation day 10.5 until birth. F1 females were mated with untreated males to obtain the F2 generation. F2 females were mated with untreated males to produce the F3 generation. In all generations, the onset of puberty, estrous cyclicity, select birth outcomes, and fertility-related indices were evaluated. In the F1 generation, prenatal DEHP exposure (200 µg, 500 mg, and 750 mg/kg/day) accelerated onset of puberty, it (200 µg/kg/day) disrupted estrous cyclicity, and it (20 µg and 200 µg/kg/day) decreased fertility-related indices. In the F2 generation, ancestral DEHP exposure (200 µg and 500 mg/kg/day) accelerated onset of puberty, it (20 and 200 µg/kg/day) disrupted estrous cyclicity, it (20 µg and 500 mg/kg/day) increased litter size, and it (500 mg/kg/day) decreased fertility-related indices. In the F3 generation, ancestral DEHP exposure (20 µg, 200 µg, and 500 mg/kg/day) accelerated onset of puberty, it (20 µg/kg/day) disrupted estrous cyclicity, and it (750 mg/kg/day) decreased female pup anogenital index. Collectively, these data indicate that prenatal DEHP exposure causes female reproductive problems in a multigenerational and transgenerational manner.

2. This is a pre-copyedited, author-produced version of an article accepted for publication in *Toxicological Sciences* following peer review. The version of record S. Rattan, E. Brehm, L. Gao, and JA. Flaws. Di(2-ethylhexyl) phthalate exposure during prenatal development causes adverse transgenerational effects on female fertility in mice. Toxicological Sciences 2018 Jun; 163(2):420-429 is available online at: https://doi.org/10.1093/toxsci/kfy042

4.2 Introduction

Phthalate esters are a family of synthetic chemicals primarily used as plasticizers to confer flexibility, prevent plastics from becoming brittle, allow products to become transparent, and improve the longevity of products [1]. Di(2-ethylhexyl) phthalate (DEHP) in particular is a synthetic, high-molecular weight, organic phthalate compound. On average, 300 million pounds of DEHP are produced annually in the United States [1]. DEHP is incorporated in a multitude of products such as personal care products, medical equipment (e.g., blood and I.V. bags), car upholstery, food and beverage containers, and building materials [2, 3]. Because DEHP is noncovalently bound, it leaches from products and into the environment [4]. Due to the frequent use of products containing DEHP, the high volume of DEHP production, and the ability of DEHP to leach from products, humans are repeatedly and continuously exposed to DEHP [3]. The average range of human exposure to DEHP is between 3 – 30 μg/kg/day [5], and studies show that 100% of human urine samples test positive for DEHP and its metabolites [6].

Phthalate exposure is higher in women than men, likely due to the presence of phthalates in personal care products and the higher use of personal care products among women compared to men [7]. In women, DEHP can be found in developmental and reproductive tissues [3]. Specifically, DEHP and its metabolites have been found in cord blood samples from newborns, breast milk from nursing mothers, and human ovarian follicular fluid. Further, DEHP and its metabolites are present in amniotic fluid from fetuses, demonstrating that DEHP has the ability to cross the placenta [3]. This is concerning because a pregnant mother exposed to DEHP also risks exposure to her developing fetus.

Of concern, DEHP acts as an endocrine disrupting chemical (EDC) and the developing fetus and the female reproductive tract are particularly susceptible to EDCs. Previous studies

have shown that prenatal exposure to DEHP adversely affects reproductive outcomes in rodents [2, 8, 9]. Specifically, our laboratory has shown that prenatal DEHP exposure (20 µg/kg/day) increased the time to pregnancy and it (750 mg/kg/day) increased the number of dead pups in the F1 generation of female mice [2]. Further, DEHP exposure (200 µg/kg/day) increased the male-to-female sex ratio in litters of the F1 generation [2]. Prenatal exposure to DEHP (200, 500, and 750 mg/kg/day) also decreased the percentage of atretic follicles in the ovary and it (20 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day) increased serum estradiol levels at PND 8 in the F1 generation [10]. In addition, another study has shown that maternal DEHP exposure accelerates the rate of follicular recruitment in the F1 generation, which could deplete the primordial follicle reserve and lead to early reproductive senescence [9, 11].

Exposure to DEHP during development may have further implications for future generations [10, 12-14]. Studies have shown that DEHP exposure during embryonic development causes transgenerational inheritance of testicular disease in male mice [15, 16]. Transgenerational effects occur when the effects of DEHP are inherited and passed through the germ-line [17, 18]. Although few studies have investigated the transgenerational effects of DEHP exposure on female reproductive outcomes, one study has shown that maternal exposure to DEHP (0.05 – 5 mg/kg/day) accelerated folliculogenesis in the ovary in the F2 and F3 generations of mice [9]. In addition, the results from another study indicated that ancestral exposure to a phthalate mixture, which included DEHP, increased uterine weight, decreased anogenital distance, and induced fertility complications in a transgenerational manner in mice [19]. Previously, our study has shown that ancestral exposure to DEHP decreased serum estradiol levels and disrupted serum progesterone levels in the F2 generation of female mice [10].

Although previous studies show that prenatal exposure to DEHP has adverse effects on ovarian follicle numbers and hormone levels, they have not assessed the effects of prenatal DEHP exposure on the onset of puberty, estrous cyclicity, and fertility [2, 9, 15]. Thus, we hypothesized that daily prenatal exposure to DEHP during the second half of pregnancy in the F0 generation adversely affects reproductive outcomes in female mice in a transgenerational manner. Specifically, we investigated whether prenatal DEHP exposure accelerates the onset of puberty, disrupts estrous cyclicity, decreases the number of pregnancies and litters, adversely affects the birth outcomes of pups, and leads to an early age of reproductive senescence in the F1, F2, and F3 generations of female mice.

4.3 Materials and Methods

Chemicals

DEHP (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of DEHP (0.022, 0.224, 560, and 840 mg/mL) were prepared by diluting DEHP in tocopherol-stripped corn oil (MP Biomedicals, Solon, OH). These stock solutions were diluted to create doses of 20 μg/kg/day, 200 μg/kg/day, 500 mg/kg/day, and 750 mg/kg/day of DEHP. DEHP concentrations were chosen based on previous studies and their environmental relevance [2, 8, 16, 20-22]. Specifically, the 20 μg/kg/day dose of DEHP was selected because the U.S. Environmental Protection Agency established the chronic oral reference dose as 20 μg/kg/day of DEHP. The reference dose is an estimate of the daily oral exposure of DEHP in the general population that has a low risk of adverse effects during the lifetime [23]. In addition, 20 μg/kg/day of DEHP falls within the estimated human exposure range based on urinary metabolite levels [5]. The 200 μg/kg/day dose of DEHP was used because it falls within the

estimated occupational range of exposure [3]. In addition, adult exposure to 200 µg/kg/day of DEHP has been shown to cause abnormal estrous cyclicity and accelerate primordial follicle recruitment in female CD-1 mice [22]. The 500 mg/kg/day dose of DEHP was selected because it has been shown to cause abnormalities in spermatogonial stem cells across multiple generations in male CD-1 mice [16]. The 750 mg/kg/day dose of DEHP was selected because adult exposure has been shown to cause abnormal estrous cyclicity and accelerate primordial follicle recruitment in adult female CD-1 mice [22].

Animals and dosing paradigm

Adult female and male CD-1 mice (Charles River, USA) were housed at 25°C in conventional polysulfone, ventilated cages on 12L:12D cycles. The mice were fed Teklad Rodent Diet 8604 (Harlan) and provided highly purified water (reverse osmosis filtered water) in polysulfone water bottles *ad libitum*. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and abide by the guidelines set forth by the National Institute of Health for the Care and Use of Laboratory Animals.

At 8 weeks of age, female mice (F0) were mated with control male mice of the same age. The female mice were monitored twice a day for the presence of a copulatory vaginal sperm plug to confirm mating. Once a copulatory vaginal sperm plug was confirmed, the presence of which was considered gestational day (GD) 0.5, the females were removed, weighed, and individually housed. Subsequently, the mice were weighed twice a week to confirm successful pregnancy. From GD 10.5 until birth of the pups, 66 pregnant dams (F0 generation) were orally dosed (7 – 27 dams/treatment group) once a day with the vehicle control (tocopherol-stripped corn oil) or with DEHP (20 µg/kg/day, 200 µg/mg/day, 500 mg/kg/day, 750 mg/kg/day) by placing a pipette

tip with the dosing solution into the cheek pouch of the mouse. This dosing regimen was selected to mimic oral exposure to DEHP in humans [1, 8, 22]. The doses were calculated and adjusted based on daily body weights, and delivered in 25 – 33 µL of tocopherol-stripped corn oil. The treatment window was chosen because it is a critical time period of ovarian development. Specifically, this is when primordial germ cells arrive at the gonad [24, 25], sex determination [26], global demethylation, and imprint erasure of primordial germ cells occurs [27].

Pregnant mice were allowed to deliver naturally and the day of birth was considered postnatal day (PND) 0. Mice born from the F0 generation were labeled the F1 generation. Female mice from the F1 generation were mated with non-treated male CD-1 mice to produce the F2 generation. Females from the F2 generation were mated with non-treated male CD-1 mice to produce the F3 generation. No mice were mated with family members.

Onset of puberty

In all generations, female mice were weaned at PND 21. One female mouse from each litter was weighed and observed daily for the presence of a vaginal opening. Once a vaginal opening was observed, the age and weight of the female were recorded, and daily vaginal lavages were performed for 30 consecutive days to monitor the onset of estrus and estrous cyclicity.

Fertility tests

In all generations, female mice were subjected to daily vaginal lavages at 3, 6, and 9 months of age for 14 consecutive days to monitor estrous cyclicity. Following 14 days of consecutive vaginal lavages, female mice were weighed and paired with non-treated male CD-1

mice to test fertility. Female mice were monitored twice a day for the presence of a copulatory vaginal sperm plug to indicate successful mating. The latency to a copulatory vaginal sperm plug was recorded. All female mice were weighed twice a week to monitor weight gain associated with pregnancy regardless of a positive copulatory vaginal sperm plug. Once a copulatory vaginal sperm plug was observed, female mice were removed from the male cage and placed individually in a fresh cage, and weight gain was continuously monitored. Female mice were paired with non-treated CD-1 male mice for a maximum of 14 days for the fertility tests. If a vaginal sperm plug was not observed, female mice were housed individually and body weight gain was continuously monitored. Body weight gain was used to monitor pregnancy loss and/or maintenance of pregnancy. The number of total females, females with a copulatory plug, pregnant females, and females that delivered pups were recorded (Table 4.1). The total number of females was defined as females paired with males. Plugged females were defined as either: a) females that had a copulatory vaginal sperm plug or b) females that did not have a copulatory vaginal sperm plug, but gained at least 4 grams of weight. Pregnant females were defined as females that either: a) had a copulatory vaginal sperm plug and gained at least 4 grams of weight during their pregnancy, b) did not have a copulatory plug, but gained at least 4 grams of weight, or c) did not have a copulatory plug, but produced a litter. Females that delivered pups were defined as dams that gave birth to either live or dead pups. These definitions were used to calculate the mating index, pregnancy rate, fertility rate, and gestational index based on the following equations used by previous studies [19, 28, 29]:

Mating index = number of females with copulatory vaginal sperm plugs/number of total females X 100

Pregnancy rate = number of pregnant females/number of total females X 100

Fertility index = number of pregnant females/number of females with copulatory vaginal sperm plugs X 100

Gestational index = number of females who delivered/number of pregnant females X 100

Once a pregnant dam gave birth to pups, all pups were counted and sexed and live pups were weighed. At PND 21, male and female body weight and anogenital distance (AGD) were recorded. The anogenital index (AGI) was calculated as the AGD divided by the cubed root of body weight [30]. Any pup deaths between PND 0 and PND 21 were recorded and reported as the percent of dead pups per litter.

Infertility during early, mid-, or late gestation was recorded for all females. Early gestation infertility was classified as no observed copulatory vaginal sperm plug, or an observed copulatory vaginal sperm plug, but no significant weight gain. Mid-gestation infertility was defined as an observed copulatory vaginal sperm plug, body weight gain around GD 7 – 12, but loss of the gained weight and a return to pre-pregnancy weight without birth of pups. Late gestation infertility was defined as a successful completion of pregnancy, but no live pups were born, suggesting infertility may be caused by late gestation issues [31].

Statistical analyses

Data were expressed as the mean ± standard error of the mean (SEM). In all generations, data from multiple female pups originating from the same litter were averaged and combined as n = 1, and data from at least 3 separate litters were used in the analyses. Data were analyzed by comparing treatment groups to control using SPSS software (SPSS Inc., Chicago, IL). Outliers were removed by the Grubb's test using GraphPad outlier calculator software (GraphPad Software Inc., La Jolla, CA). Two cohorts (two groups of animals) were mated to produce the F3

generations. To test for cohort differences, data were tested using a general linear model univariate test. If there was an interaction between treatment and cohort in tests of between-subjects effects, then the data from the first cohort was analyzed. If no interaction effect between treatment and cohort occurred, then both cohorts were analyzed together. Data that were continuous were assessed for normal distribution by Shapiro-Wilk analysis. If data met assumptions of normal distribution and homogeneity of variance, data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett (2-sided) *post-hoc* comparisons. If data were presented as percentages, were not normally distributed, and/or did not meet homogeneity of variance assumptions, the independent sample Kruskal-Wallis H followed by Mann-Whitney U non-parametric tests were performed. Data that were nominal were analyzed using the one-tailed Fisher's Exact test to compare individual treatment groups to control. For all comparisons, statistical significance was determined by a p-value ≤ 0.05. In instances in which p-values were greater than 0.05, but less than 0.1, data were considered to exhibit a trend towards significance.

4.4 Results

Effects of DEHP exposure on pubertal body weights, age of vaginal opening, and age of first estrus

To monitor puberty, the body weight at weaning, body weight at vaginal opening, age at vaginal opening, and age at first estrus were recorded. Our results indicate that in the F1, F2, and F3 generations, prenatal and ancestral exposure to DEHP did not affect the body weight of female mice at weaning (PND 21) (Figure 4.1A) or the body weight of female mice at vaginal opening (Figure 4.1B).

In the F1 generation, prenatal exposure to the 200 µg/kg/day dose of DEHP accelerated the age at vaginal opening, but this change was of borderline statistical significance compared to controls (Figure 4.1C, n = 5 – 16 dams/treatment group; $p = 0.061$). In contrast, exposure to the 200 µg/kg/day dose of DEHP significantly accelerated the age at first estrus compared to controls (Figure 4.1D, n = 6 – 15 dams/treatment group; $p \leq 0.05$). In the F2 generation, ancestral exposure to DEHP did not significantly affect the age at vaginal opening (Figure 4.1C), but the 500 mg/kg/day dose of DEHP significantly accelerated the age at first estrus compared to controls (Figure 4.1D, n = 11 – 15 dams/treatment group; $p \leq 0.05$). In the F3 generation, ancestral exposure to the 20 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day doses of DEHP significantly accelerated the age at vaginal opening compared to controls (Figure 4.1C, n = 7 – 11 dams/treatment group; $p \leq 0.05$), but DEHP exposure did not affect the age at first estrus (Figure 4.1D).

Effects of DEHP exposure on post-pubertal cyclicity

To examine the effects of DEHP exposure on estrous cyclicity following the onset of puberty, estrous cyclicity was monitored for 30 consecutive days following vaginal opening. In the F1 generation, prenatal exposure to the 750 mg/kg/day dose of DEHP increased the percent of time in proestrus and the 200 µg/kg/day dose of DEHP increased the percent of time in estrus and decreased the percent of time in diestrus compared to controls, but the increases were borderline statistically significant (Figure 4.1E, n = 6 – 15 dams/treatment group; $p = 0.068$ for the increased percent of time in proestrus in the 750 mg/kg/day group vs. control; $p = 0.078$ for the increased percent of time in estrus in the 200 µg/kg/day group vs. control; $p = 0.070$ for the decreased percent of time in diestrus in the 200 µg/kg/day group vs. control). In the F2

generation, ancestral exposure to the 200 µg/kg/day and 500 mg/kg/day doses of DEHP increased the percent of time in estrus and decreased the percent of time that the mice spent in diestrus compared to controls (Figure 4.1E, n = 11 – 15 dams/treatment group; $p \leq 0.05$ for the percent of time in diestrus in the 200 µg/kg/day group vs. control and for the percent of time in estrus and diestrus in the 500 mg/kg/day group vs. control; $p = 0.078$ for the percent of time in estrus 200 µg/kg/day group vs. control). In the F3 generation, ancestral DEHP exposure did not affect post-pubertal estrous cyclicity (Figure 4.1E).

Effects of DEHP exposure on estrous cyclicity

To examine the effects of DEHP exposure on estrous cyclicity during adult life, estrous cyclicity was monitored for 14 consecutive days at 3, 6, and 9 months of age. In the F1 generation, prenatal exposure to DEHP did not affect estrous cyclicity at 3 or 6 months of age, but the 500 mg/kg/day dose of DEHP borderline significantly increased the percent of time that mice spent in proestrus and the 200 µg/kg/day dose of DEHP significantly increased the percent of time that mice spent in estrus compared to controls at 9 months of age (Figure 4.2A, n = 4 – 7 dams/treatment group; $p = 0.071$ for the 500 mg/kg/day group vs. control; $p \leq 0.05$ for the 200 µg/kg/day group vs. control). In the F2 generation, ancestral exposure to DEHP did not affect estrous cyclicity at 3 or 6 months of age. However, the 20 µg/kg/day dose of DEHP significantly increased the percent of time that the mice spent in estrus and the 200 µg/kg/day dose significantly decreased the amount of time that the mice spent in diestrus compared to controls at 9 months of age (Figure 4.2B, n = 10 – 15 dams/treatment group; $p \leq 0.05$). Further, the 200 µg/kg/day dose of DEHP increased the time that the mice spent in estrus and the 20 µg/kg/day dose of DEHP decreased the that time the mice spent in diestrus at 9 months of age, but these

changes were borderline statistically significant (Figure 4.2B, n = 10 – 15 dams/treatment group; p = 0.086 for the 200 µg/kg/day group vs. control; p = 0.066 for the 20 µg/kg/day group vs. control). In the F3 generation, ancestral DEHP exposure did not affect estrous cyclicity at 3 months of age, but the 20 µg/kg/day dose of DEHP significantly increased that the percent of time mice spent in estrus and decreased the percent of time that mice spent in diestrus compared to controls at 6 months of age (Figure 4.2C, n = 9 – 10 dams/treatment group; $p \leq 0.05$). Further, the 20 µg/kg/day dose of DEHP decreased the time that the mice spent in diestrus at 9 months of age, but this was of borderline significance (Figure 4.2C, n = 0 dams/treatment group; p = 0.089).

Effects of DEHP exposure on birth outcomes

Select birth outcomes such as litter size, percentage of female pups, and mortality rates were monitored during the fertility tests. In the F1 and F3 generations, exposure to DEHP did not affect the litter size compared to controls. However, in the F2 generation, ancestral exposure to the 20 µg/kg/day and 500 mg/kg/day doses of DEHP increased the litter size compared to controls (Figure 4.3A, n = 7 – 26 dams/treatment group; $p \leq 0.05$).

In the F1 and F2 generations, prenatal and ancestral exposure to DEHP did not affect the percentage of female pups. However, in the F3 generation, ancestral exposure to the 20 µg/kg/day dose of DEHP increased the percentage of female pups per litter, but the increase only trended towards significance (Figure 4.3B, n = 24 – 26 dams/treatment group; p = 0.080). In addition, in the F1, F2, and F3 generations, exposure to DEHP not affect the pup mortality rate compared to controls (Figure 4.3C).

Effects of DEHP exposure on anogenital index

In the F1 and F2 generations, prenatal and ancestral DEHP exposure did not affect female pup anogenital index compared to controls (Figure 4.4A). However, in the F3 generation, ancestral exposure to the 750 mg/kg/day dose of DEHP decreased the female pup anogenital index in the 3 month old litter compared to controls (Figure 4.4A, n = 5 – 10 dams/treatment group; $p \leq 0.05$), but it did not affect the female anogenital index in the 6 or 9 month old litters. In the F1, F2, and F3 generations, prenatal and ancestral exposure to DEHP did not affect the male pup anogenital index compared to controls (Figure 4.4B).

Effects of DEHP exposure on fertility-related indices

Exposure to DEHP did not affect the mating index in any generations. Specifically, the mating index ranged from 94 – 100 % in all treatment groups in the F1, F2, and F3 generations (Figure 4.5A). In the F1 generation, prenatal exposure to the 20 µg/kg/day and 200 µg/kg/day doses of DEHP decreased the pregnancy rate compared to controls, but the decrease only trended towards statistical significance (Figure 4.5B, n = 37 – 60 dams/treatment group; $p = 0.082$ and 0.069, respectfully). Ancestral DEHP exposure did not significantly affect the pregnancy rate in the F2 or F3 generations. In the F1 generation, prenatal exposure to the 20 µg/kg/day and 200 µg/kg/day doses of DEHP decreased the fertility index compared to controls (Figure 4.5C, n = 33 – 58 dams/treatment group; $p = 0.074$ and $p \leq 0.05$, respectfully). In contrast, ancestral exposure to DEHP did not affect the fertility index in the F2 or F3 generations. In the F1 and F3 generations, prenatal and ancestral exposure to DEHP did not affect the gestational index. However, in the F2 generation, ancestral exposure to the 500 mg/kg/day dose of DEHP

significantly decreased the gestational index compared to controls (Figure 4.5D, n = 41 – 44 dams/treatment group; $p \leq 0.05$).

Effects of DEHP exposure on types of infertility

In the F1 generation, prenatal exposure to the 20 µg/kg/day dose of DEHP increased the number of dams with early gestation infertility and the 200 µg/kg/day dose of DEHP increased the number of dams with early and mid-gestation infertility compared to controls (Table 4.2). In the F2 generation, ancestral exposure to the 500 mg/kg/day dose of DEHP increased the number of dams with mid-gestation infertility compared to controls (Table 4.2). In the F3 generation, ancestral DEHP exposure did not appear to change the types of infertility compared to controls (Table 4.2).

4.5 Discussion

Our data indicate that prenatal and ancestral exposure to DEHP negatively impacts reproductive outcomes across the F1, F2, and F3 generations of mice (Figure 6). Specifically, DEHP exposure accelerated puberty, interfered with normal estrous cyclicity, increased litter size, decreased anogenital index in female offspring, and decreased pregnancy rate, fertility index, and gestational index in multigenerational and transgenerational manners (Figure 6).

It is interesting that prenatal DEHP exposure did not produce a linear dose response. DEHP is a known EDC, and a characteristic of EDCs is that the effects are not proportional to the dose and therefore, often do not follow a linear relationship [32]. It is also important to note that the effects of DEHP exposure were not always the same in each generation. The different effects of DEHP exposure in each generation are likely due to the different developmental

windows of exposure. During prenatal DEHP exposure, the F1 generation was exposed as the developing pup, the F2 generation was exposed as the developing ovaries within the F1 pup, and the F3 generation was not directly exposed to DEHP. Therefore, the effects of DEHP exposure in the F3 generation are considered transgenerational.

Our data showed that DEHP exposure accelerated the onset of puberty in all three generations. It is likely that the ability of prenatal and ancestral DEHP exposure to accelerate puberty is not through a mechanism involving body weight because our data indicated that prenatal and ancestral DEHP exposure did not affect body weight. Further, it is likely that prenatal and ancestral exposure to DEHP did not accelerate the onset of puberty through the alteration of serum sex steroid hormone levels. Previously, our laboratory demonstrated that prenatal and ancestral exposure to DEHP did not significantly affect serum estradiol levels at PND 21, an age earlier than the observed pubertal onset [10]. Although we previously showed that ancestral DEHP (20 µg/kg/day and 500 mg/kg/day) exposure increased serum progesterone levels at PND 21 in the F2 generation [10], serum estradiol, but not progesterone, is critical for the onset of puberty [33, 34]. Although we did not detect differences in serum estradiol at PND 21, it is possible that serum estradiol levels at other time points impacted the onset of puberty.

One study demonstrated that ancestral exposure to a plasticizer mixture consisting of 750 mg/kg/day of DEHP accelerated the age at vaginal opening in the F3 generation in rats [15]. However, our data differ from Manikkam *et al*. because that study showed that the mixture consisting of 750 mg/kg/day delayed vaginal opening in the F1 generation, whereas we did not observe a delay in vaginal opening in the 750 mg/kg/day DEHP dose group in the F1 generation. The reasons for differences between our F1 generation data and those from Manikkam *et al*. are

unclear, but they may be due to species differences between mice and rats or interactions between chemicals in the mixture that produced different effects than DEHP alone.

In addition, our data showed that prenatal and ancestral exposure to DEHP disrupted post-pubertal cyclicity in the F1 generation and disrupted post-pubertal cyclicity and adult estrous cyclicity in the F2 and F3 generations. Our results agree with previous studies showing that adult exposure to DEHP (20 µg/kg/day) increased the percentage of days that the mice spent in estrus [8]. Previously, our laboratory showed that ancestral exposure to DEHP (200 µg/kg/day, 500, and 750 mg/kg/day) decreased serum progesterone levels during the diestrus phase in the F2 generations [10]. Our current study showed that mice exposed to DEHP spent more time in diestrus when basal levels of progesterone are normally low [35]. Thus these data are consistent with our previous data showing that ancestral DEHP exposure decreased serum progesterone levels. Further, rodents undergoing reproductive senescence will experience a state of persistent estrus followed by complete acyclicity, or a chronic state of diestrus [36]. Therefore, our data suggest that ancestral DEHP exposure may accelerate the onset of reproductive senescence in the F2 and F3 generations and that it can have lasting effects on estrous cyclicity in both a multigenerational and transgenerational manner.

Ancestral exposure to DEHP increased litter size in the F2 generation. These data were unexpected because previously we showed that ancestral exposure to DEHP did not affect the number of corpora lutea in mice [10]. Corpora lutea are used as indicators of ovulation, and no change in the number of corpora lutea suggests normal ovulation rates, and subsequently, normal litter sizes. However, this is the opposite of what was observed in the present study. Interestingly, previous literature has shown that direct exposure to DEHP (250 and 500 mg/kg/day) reduced the number and size of corpora lutea in mice [37]. It is possible that direct

exposure to DEHP and ancestral exposure to DEHP impacts ovulation differently. The different ovulation rates may explain the difference in the results in our study versus the previous study. However, future studies should investigate the mechanism by which ancestral DEHP exposure increased litter size without affecting the number of corpora lutea.

Ancestral exposure to DEHP increased the female to male sex ratio in litters produced by the F3 generation. Although Niermann *et al.* showed that prenatal exposure to DEHP (750 mg/kg/day) increased the male to female sex ratio in litters produced by the F1 generation [2], we observed a different trend in the F3 generation. Although the increase in the percentage of female pups per litter was only borderline statistically significant ($p = 0.080$), it appears that our control in the F3 generation was much lower than the expected 50% of female pups. The lower percentage of females in the control group may have influenced the observed borderline increase in percentage of female pups per litter observed in the F3 generation. In addition, random variation may have influenced the increased female to male ratio.

Our data indicate that ancestral exposure to DEHP decreased female AGI at 3 months of age in the litters produced by the F3 generation, revealing a transgenerational effect of DEHP exposure on AGI. AGI is an indicator of androgen exposure to the fetus *in utero*. It is possible that androgen levels were decreased in the F3 mothers, therefore decreasing the AGI of the female pups, however future studies should test this hypothesis. Our findings are in contrast to another study that showed that maternal ancestral DEHP exposure (5 µg/kg/day) increased AGI in the F3 generation of female mice [38]. It is likely that our results differ from that study because we used different doses of DEHP. DEHP exerts non-monotonic dose responses, and therefore, different doses may affect the AGI differently. Further, the previous study observed a decrease in body weight in the 5 µg/kg/day pups, which likely drove the significant effects on

AGI [38]. However, in our study, we did not observe a significant difference in the body weights of the pups produced at 3 months of age in the F3 generation (data not shown).

The different fertility-related indices were calculated based on if a female mated, became pregnant, and gave birth. Our data indicate that DEHP exposure does not affect the mating index. Our data agree with previous studies that have shown that prenatal exposure to a phthalate mixture that included DEHP did not impact the mating index [19, 39]. In contrast, Quinnies *et al*. demonstrated that prenatal exposure to DEHP at 400 µg/kg/day caused females to be less interactive and potentially less interested in mating with males [38]. A lack of interest in mating with males should produce a low mating index, however, we did not observe this in our study. It is possible that inbred strain (C57BL/6J) used in the Quinnies *et al*. study and outbred strain (CD-1) used in our study contribute to the observed differences in results [40, 41].

Our data indicate that prenatal exposure to DEHP reduced the pregnancy rate in the F1 generation. The reduced pregnancy rate, but lack of an impact on mating index, indicates that DEHP exposure does not affect the ability of the mice to mate, but it affects the ability of the mice to become pregnant. Further, DEHP reduced the fertility index in the F1 generation, suggesting that DEHP decreases the number of females that became pregnant after mating. The exact mechanisms by which DEHP interferes with pregnancy and fertility are unknown. It is possible that DEHP exposure targets the ovary because DEHP exposure has been shown to decrease the ovarian reserve and increase atretic follicles in mice [8, 9, 42]. Additionally, it is possible that DEHP exposure interferes with uterine functions as indicated by previous literature demonstrating that exposure to a phthalate mixture, including DEHP (20 and 200 µg/kg/day phthalate mixture), increases uterine weight in young mice [39]. In our previous study, prenatal exposure to DEHP increased serum estradiol levels early in life [10], and this increase persisted

at one year of age in the F1 generation [43]. Although this increase in estradiol was not at the same age as the observed decreases in the pregnancy and fertility rates in the F1 generation, it is possible that estradiol levels were affected throughout life and impacted these fertility-related indices because estradiol is critical for normal fertility.

Interestingly, ancestral DEHP exposure reduced the gestational index in the F2 generation. The data indicate that although the females successfully mated and became pregnant, they had difficulty carrying the pregnancy to term in the F2 generation. The exact mechanism by which ancestral DEHP exposure affects the maintenance of pregnancy is not known, but previous literature has shown that exposure to DEHP (250 and 500 mg/kg/day) during pregnancy inhibited vascularization of corpora lutea and increased corpora lutea regression, suggesting that it might increase the risk of miscarriage by suppressing luteal function [37]. The potential for DEHP to cause miscarriages is supported by our data indicating that ancestral DEHP exposure increased mid-gestation infertility in the F2 generation. Further, the potential of DEHP to cause miscarriages is supported by a study showing that DEHP exposure (250 and 500 mg/kg/day) is teratogenic and has a lethal impact on the mouse fetus [44]. Further, we have previously shown that ancestral exposure to DEHP decreased serum progesterone levels in young adult mice [10] and at one year of age in the F2 generation [43]. Progesterone is important for thickening the lining of the uterus and maintaining a pregnancy [45]. Thus, DEHP-induced low levels of progesterone may contribute to the reduced gestational index observed in the F2 generation of our study.

In our study, the F0 dams were orally exposed to DEHP, but previous studies have shown that the bioactive metabolite, MEHP, is responsible for the toxic effects [20, 46]. Therefore, it is likely that the bioactive metabolite, MEHP, affected the observed onset of puberty, estrous

cyclicity, and fertility related indices. In our study, we did not measure serum metabolite levels of DEHP in the F0 dams. However, future studies on the toxicokinetics of DEHP exposure are important and would be useful for comparison to human exposure levels.

Collectively, our results indicate that prenatal DEHP exposure induced some multigenerational and transgenerational effects on female reproductive outcomes. Multigenerational effects on female reproductive outcomes were observed in the onset of puberty, estrous cyclicity, litter size, and fertility-related indices. Transgenerational effects on female reproductive outcomes were observed in the onset of puberty, estrous cyclicity, and anogenital index. Future studies should investigate the underlying mechanisms of the DEHP-induced effects on reproductive outcomes in the F1, F2, and F3 generations. Given that DEHP is quickly metabolized and cleared from the body soon after birth [5, 47, 48] and the effects are observed in the adults of the F1 generation and into the F2 and F3 generations, these observations suggest that DEHP exposure causes toxicity via epigenetic mechanisms. Future studies should investigate whether ancestral exposure to DEHP influences the function of the ovary and if epigenetic changes contribute to the decrease in reproductive outcomes observed in our study. Further, recently, the use of DEHP replacements have steadily increased over the years [49, 50]. While phasing out DEHP would be ideal, few studies have investigated the effects of DEHP replacements on female reproduction. Therefore, we suggest that future studies investigate the effects of DEHP replacements on female reproduction before replacing DEHP with a chemical of unknown toxicity.

4.6 Tables, Figures, and Legends

Table 4.1 The number of mice used for the fertility related indices

Generation		Treatment				
		Control	DEHP 20 µg/kg/day	DEHP 200 µg/kg/day	DEHP 500 mg/kg/day	DEHP 750 mg/kg/day
F1	Total Female	60	39	45	41	29
	Plugged Female	58	37	44	41	29
	Pregnant Female	57	33	38	41	26
	Delivered Female	54	31	35	38	23
F2	Total Female	46	35	31	45	21
	Plugged Female	44	33	30	43	20
	Pregnant Female	41	31	29	43	18
	Delivered Female	38	30	29	33	17
F3	Total Female	14	34	30	19	31
	Plugged Female	14	32	29	19	30
	Pregnant Female	13	32	28	18	30
	Delivered Female	12	29	28	17	28

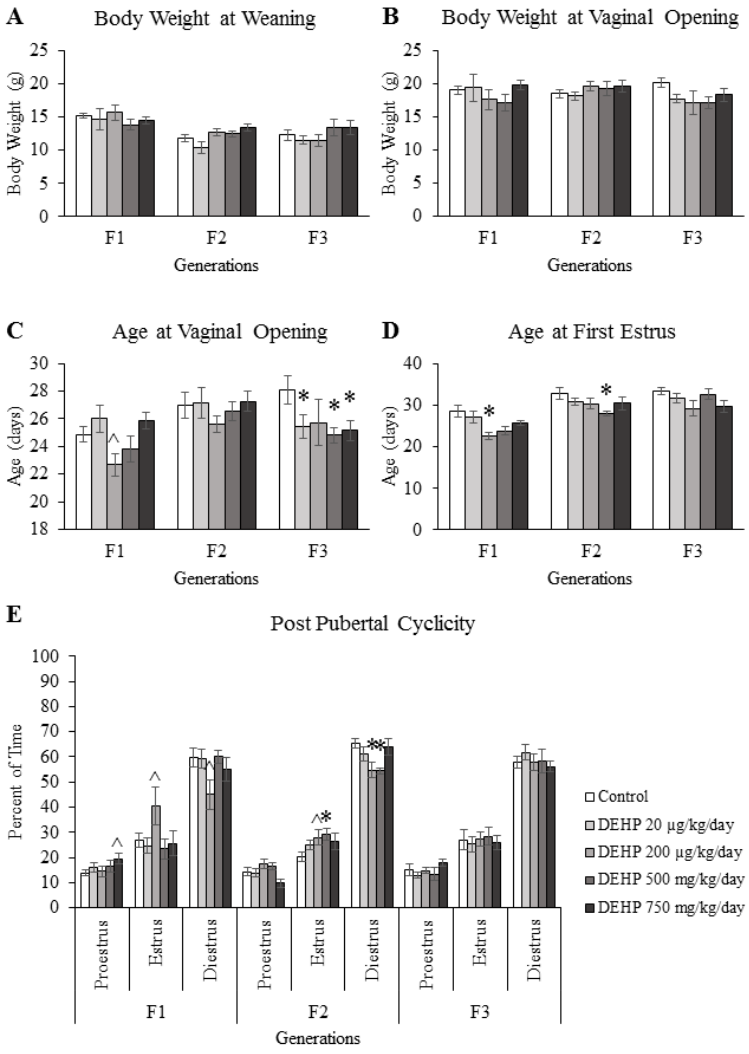
The number of females mated, number of females with copulatory plugs, number of pregnant females, and number of females that delivered pups recorded in the F1, F2, and F3 generations.

Table 4.2 Different types of infertility in the F1 – F3 generations

Generation	Type of Infertility	Treatment				
		Control	DEHP 20 µg/kg/day	DEHP 200 µg/kg/day	DEHP 500 mg/kg/day	DEHP 750 mg/kg/day
F1	Early Gestation	3	6	5	0	3
	Mid-Gestation	3	3	5	3	2
	Late Gestation	0	0	1	2	2
F2	Early Gestation	6	3	2	1	2
	Mid-Gestation	2	2	0	11	2
	Late Gestation	0	0	0	0	2
F3	Early Gestation	3	2	1	1	4
	Mid-Gestation	2	1	1	3	1
	Late Gestation	0	1	1	0	0

The number of female mice that experience infertility in early, mid-, or late gestation in the F1, F2, and F3 generations.

Figure 4.1 The effects of DEHP exposure on pubertal outcomes in the F1 – F3 generations



The effects of prenatal exposure to DEHP on pubertal outcomes in the F1, F2, and F3 generations. The body weight at weaning (A), body weight at vaginal opening (B), age at vaginal opening (C), age at first estrus (D), and post pubertal cyclicity (E) are shown for the F1, F2, and F3 generations. Graphs represent mean ± standard error of the mean from 5 – 16 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); $0.05 < \text{^}p \leq 0.078$ (borderline difference compared to the control).

Figure 4.2 The effects of DEHP exposure on estrous cyclicity for the F1 – F3 generations

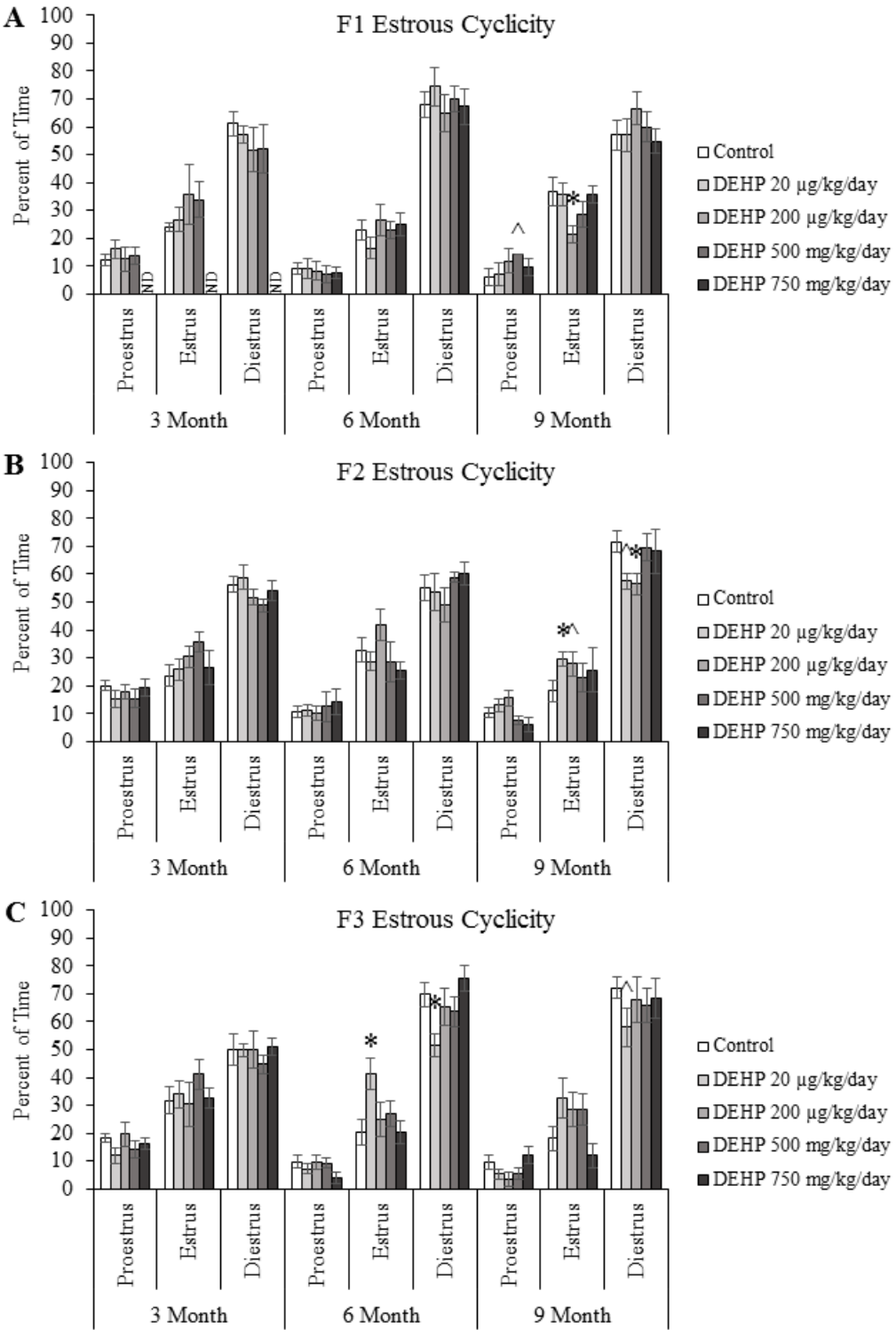
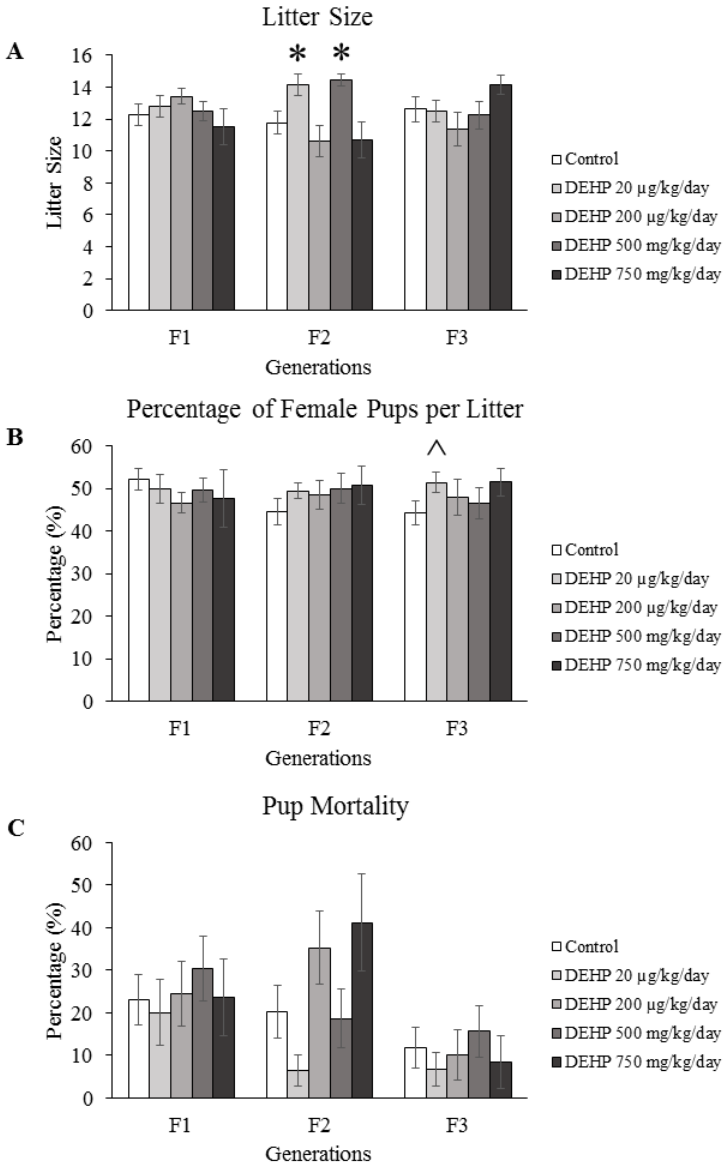


Figure 4.2 (cont.)

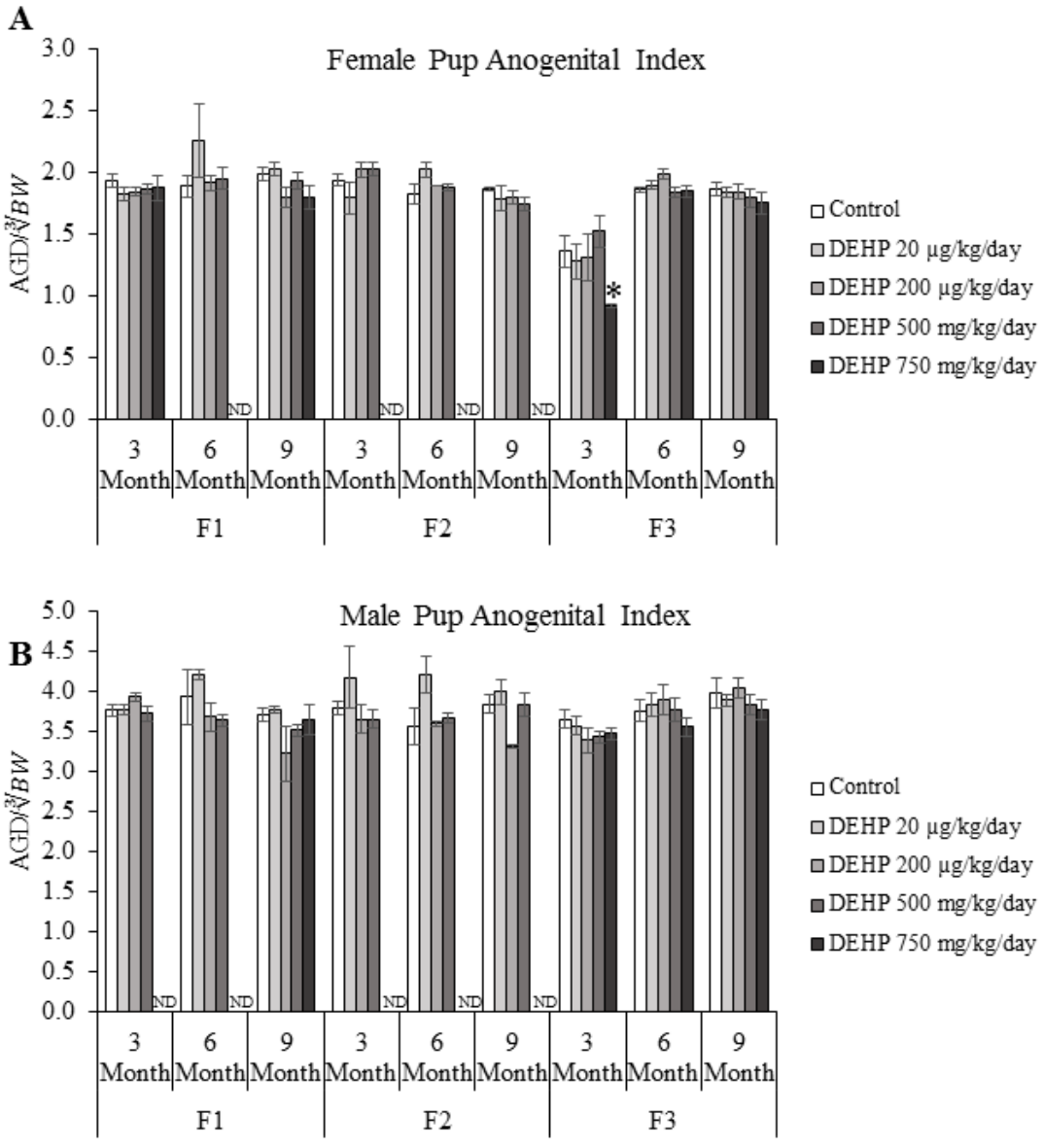
The effects of prenatal exposure to DEHP on estrous cyclicity are shown for the F1 generation (A), F2 generation (B), and F3 generation (C) at 3, 6, and 9 months of age. Graphs represent mean ± standard error of the mean from 2 – 15 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); $0.05 < {}^{\wedge}p \leq 0.086$ (borderline difference compared to the control); ND = no data.

Figure 4.3 The effects of DEHP exposure on birth outcomes in the F1 – F3 generations



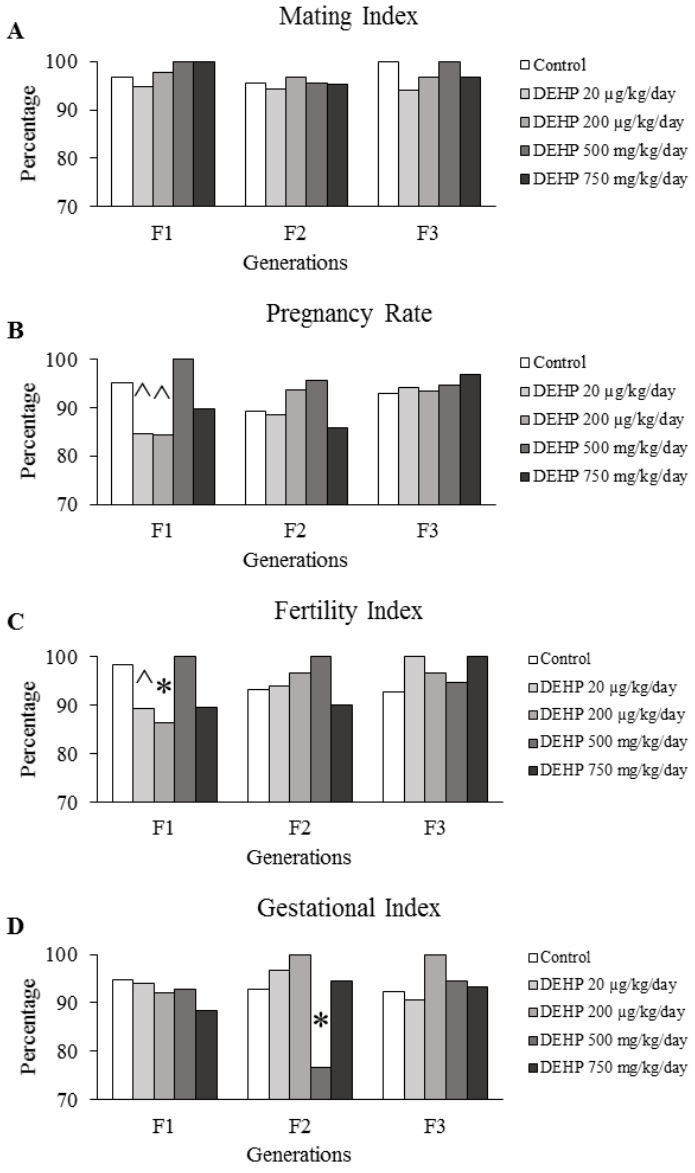
The effects of prenatal exposure to DEHP on birth outcomes such as litter size (A), percentage of females per litter (B), and mortality rate (C) are shown for the F1, F2, and F3 generations. Graphs represent mean ± standard error of the mean from 7 – 44 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); ^$p = 0.080$ (borderline difference compared to the control).

Figure 4.4 The effects of DEHP exposure on female pup anogenital index in the F1 – F3 generations



The effects of prenatal exposure to DEHP on female pup anogenital index (A) and male pup anogenital index (B) are shown for the F1, F2, and F3 generations at 3, 6, and 9 months of age. Graphs represent mean ± standard error of the mean from 2 – 13 dams per treatment group. *$p \leq 0.05$ (significant difference compared to the control); ND = no data.

Figure 4.5 The effects of DEHP exposure on fertility related indices in the F1 – F3 generations



The effects of prenatal exposure to DEHP on the fertility related indices the F1, F2, and F3 generations. The mating index (A), pregnancy rate (B), fertility index (C), and gestational index (D) are represented. Graphs represents mean ± standard error of the mean from 12 – 60 dams per treatment group. * $p \leq 0.05$ (significant difference compared to the control); $0.05 < {}^{\wedge}p \leq 0.082$ (borderline difference compared to the control).

Figure 4.6 The effects of DEHP exposure on female fertility summarized

	F1	F2	F3
Puberty	Early Onset (200 µg/kg/day)	Early Onset (500 mg/kg/day)	Early Onset (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day)
Estrous Cyclicity	Abnormal (200 µg/kg/day, 500 mg/kg/day)	Abnormal (20 µg/kg/day, 200 µg/kg/day)	Abnormal (20 µg/kg/day)
Litter Size		Increased (20 µg/kg/day, 500 mg/kg/day)	
Percentage Female			Increased (20 µg/kg/day)
Anogenital Index			Decreased (750 mg/kg/day)
Pregnancy Rate	Decreased (20 µg/kg/day, 200 µg/kg/day)		
Fertility Index	Decreased (20 µg/kg/day, 200 µg/kg/day)		
Gestational Index		Decreased (500 mg/kg/day)	

The effects of DEHP on female fertility at each dose are summarized for the F1, F2, and F3 generations. The figure summarizes the main finding from the study.

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CHAPTER 5

Prenatal and Ancestral Exposure to Di(2-ethylhexyl) Phthalate Alters Gene Expression and DNA Methylation in Mouse Ovaries

5.1 Abstract

Di(2-ethylhexyl) phthalate is a commonly used plasticizer in polyvinyl chloride products to induce flexibility and stability in plastic products. However, DEHP is a known endocrine disrupting chemical and studies show that it causes transgenerational reproductive toxicity in female rodents. However, the mechanism of action in the F3 generation is not understood. Therefore, the purpose of this study was to determine the effects of prenatal and ancestral DEHP exposure on various ovarian pathways critical for cell growth, proliferation, and function, and to determine the effects on DNA methylation pathways in mouse ovaries from the F1, F2, and F3 generations. Pregnant CD-1 dams were orally exposed to tocopherol-stripped corn oil (vehicle control) or DEHP (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, or 750 mg/kg/day) daily from gestation day 10.5 until birth. Once the pregnant dams gave birth to the F1 generation, DEHP exposure ceased. Female mice from the F1 generation were mated with non-treated male mice to produce the F2 generation. Females from the F2 generation were used to produce the F3 generation. At postnatal day 21 for each generation, mice were euthanized and ovaries were removed for gene expression analysis of various ovarian pathways via qPCR and 5-methyl cytosine (5-mC) quantification. The results show that in the F1 generation, prenatal DEHP exposure disrupted the expression of cell cycle regulators, decreased the expression of peroxisome-proliferator activating receptors, and increased the percentage of 5-mC in the ovary compared to control. In the F2 generation, exposure to DEHP decreased the expression of steroidogenic enzymes, dysregulated the expression of PI3K-pathway factors, decreased the

expression of apoptosis factors, and decreased the expression of *Tet* compared to controls. In the F3 generation, ancestral DEHP exposure decreased the expression of steroidogenic enzymes, decreased the expression of PI3K-pathway factors, decreased the expression of cell cycle regulators, decreased the expression of apoptosis factors, decreased the expression of *Esr2*, decreased the expression of DNA methylation factors, and decreased the percentage of 5-mC compared to controls. Overall, the data show that prenatal and ancestral DEHP greatly suppresses gene expression of pathways required for folliculogenesis and steroidogenesis in the ovary in a transgenerational manner and that gene expression may be in influenced by DNA methylation. These results provide insight into some of the mechanisms of DEHP-mediated toxicity in the ovary across generations. Supported by NIH P01 ES022848, EPA RD-83459301, T32 ES007326, F31 ES030467, and the Billie A. Field Fellowship.

5.2 Introduction

Phthalates are a family of synthetic chemicals that act as plasticizers to confer flexibility and prevent plastics from becoming brittle [1]. Phthalates are critical for the production of consumer goods. Many types of phthalates exist, but di(2-ethylhexyl) phthalate (DEHP) is a common plasticizer found in polyvinyl chloride products. DEHP is incorporated into a multitude of products including personal care products, medical equipment (i.e., blood and I.V. bags), car upholstery, food and beverage containers and packaging, and building materials, particularly vinyl products [1-3]. DEHP is noncovalently bound to the polymer chains within these products; therefore, DEHP may migrate from the products and into the environment after repeated use, heating, and cleaning [4]. Humans are exposed to DEHP by oral ingestion, inhalation, and dermal contact. However, the most common route of exposure to DEHP and phthalates in

general is by ingestion. The estimated range of human exposure to DEHP is between 3 – 30 µg/kg/day [2, 5-7]. Human urine samples persistently test positive for DEHP and its metabolites, indicating that humans are repeatedly and continuously exposed to DEHP [8]. This is further supported by the detection of DEHP in human tissues such as blood, amniotic fluid, umbilical cord blood, breast milk, and ovarian follicular fluids in humans [2, 9-12].

DEHP is an endocrine disrupting chemical (EDC) [2, 13, 14], and reproductive tissues such as the gonads are particularly susceptible to EDCs. In humans, *in utero* exposure to DEHP is associated with decreased free testosterone and free testosterone:estradiol ratio the in cord blood of both male and female newborns [15, 16]. DEHP exposure has been shown to interfere with obstetrical outcomes, puberty, and gonadal function [17]. DEHP exposure is also associated with an early age of pubic hair development in young girls, an indicator of precocious puberty [18]. Further, *in utero* exposure to DEHP metabolites is associated with an earlier age of menarche, or first menstruation in young girls [19]. Finally, urinary concentrations of DEHP metabolites are negatively associated with total oocytes, mature oocytes, fertilized oocytes, and top quality embryos, indicating that DEHP impairs oocyte parameters [20].

The ability of DEHP to cause adverse reproductive outcomes is a major concern for the F1 and subsequent generations. Changes in the ovary may be inherited by the subsequent generations through the female germ cells [21]. Transmission of disease due to direct prenatal DEHP exposure may cause multigenerational effects and ancestral exposure to DEHP may cause transgenerational effects in the F3 generation. This is because during a developmental exposure window, the pregnant mouse (F0 generation) is exposed to DEHP via ingestion. Therefore, the F1 generation is directly exposed to DEHP as a fetus, and the F2 generation is directly exposed to DEHP as the developing germ cells in the F1 fetus, causing multigenerational effects in the F1

and F2 generations [22]. Effects observed in the F3 generation are not due to direct exposure, but instead are due to ancestral exposure, meaning they are transgenerational effects [22].

Experimental studies show that DEHP exposure causes numerous multigenerational and transgenerational phenotypes in female reproduction. Specifically, prenatal DEHP exposure decreases the percentage of dams that give birth in the F2 generation of mice [23]. Ancestral DEHP exposure accelerates the onset of puberty and reproductive senescence in the F3 generation of female mice [23, 24]. Further, DEHP exposure during prenatal development dysregulates folliculogenesis, alters sex steroid hormone levels, and increases the presence of ovarian cysts in a multigenerational manner [24, 25]. In addition, ancestral exposure to DEHP accelerates early folliculogenesis in a transgenerational manner [25]. Although studies demonstrate that phthalate exposure causes transgenerational effects on the ovary, the mechanisms underlying these effects are not well understood.

Epigenetic modification is thought to be the mechanism by which transgenerational effects are inherited [26]. Epigenetics are mitotically and meiotically heritable changes in gene function, without changing DNA sequences [27, 28]. These heritable changes in the epigenome define and control cell and tissue development by controlling gene expression [29]. Multiple molecular mechanisms alter the epigenome, however, DNA methylation is the most commonly studied epigenetic mechanism [30]. DNA methylation patterns are placed and removed by DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) enzymes [31-33]. DNMTs are a family of enzymes that methylate CpG dinucleotides in DNA. DNMT1 is the maintenance DNMT that maintains original DNA methylation patterns in a cell lineage; it methylates CpG sites during DNA replication so that both daughter cells have the same DNA methylation patterns [34-36]. DNMT3A and DNMT3B methylate CpG sites on naked DNA outside of DNA

replication and are required for genome-wide de novo methylation [31]. TETs are enzymes that oxidize 5-methyl cytosine (5-mC) as a demethylation mechanism [32, 37]. TET1 is primarily responsible for oxidizing 5-mC into 5-hydroxymethyl cytosine (5-hmC), whereas TET2 and TET3 primarily oxidize 5-hmC into further oxidized versions which are eventually replaced with an unmethylated, unmodified cytosine [32, 38].

Studies have demonstrated that DEHP exposure modulates DNA methylation. Specifically, prenatal DEHP exposure induces a long-lasting and robust promoter methylation-related silencing of fundamental genes in sperm physiology [39]. Further, *in utero* DEHP exposure is associated with an enrichment of DNA methylation levels of genes involved in the androgen response, estrogen response, and spermatogenesis [40]. Prenatal exposure to DEHP differentially expressed 406 genes related to reproductive processes in rat ovaries [41]. These studies indicate that DEHP acts through methylation, however, these studies do not indicate if the effects of DEHP on methylation status continue throughout generations. Therefore, the current study was designed to evaluate the pathways and mechanisms by which prenatal and ancestral exposure to DEHP influence key ovarian functions in the F1, F2, and F3 generations of mice. Specifically, this study tested the hypothesis that prenatal and ancestral DEHP exposure disrupt ovarian functions via DNA methylation and subsequently alters gene expression of several ovarian pathways critical for cell growth, proliferation, and function (i.e., the sex steroid hormone synthesis pathway, phosphoinositide 3-kinase pathway (PI3K), cell cycle regulators, apoptosis and oxidative stress factors, steroid hormone receptors, and insulin-like growth factors) [25, 42-49] and DNA methylation effectors such as DNMTs and TET enzymes.

5.3 Materials and Methods

Chemicals

DEHP (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of DEHP (0.022, 0.224, 560, and 840 mg/mL) were prepared by diluting DEHP in tocopherol-stripped corn oil (MP Biomedicals, Solon, OH). These stock solutions were diluted to create doses of 20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day of DEHP. DEHP concentrations were chosen based on previous studies and their environmental relevance [45, 46, 50-53]. Specifically, the 20 µg/kg/day dose of DEHP was selected because the U.S. Environmental Protection Agency established the chronic oral reference dose as 20 µg/kg/day of DEHP. The reference dose is an estimate of the daily oral exposure of DEHP in the general population that has a low risk of adverse effects during the lifetime [54]. In addition, 20 µg/kg/day of DEHP falls within the estimated human exposure range based on urinary metabolite levels [5]. The 200 µg/kg/day dose of DEHP was used because it falls within the estimated occupational range of exposure [2]. In addition, adult exposure to 200 µg/kg/day of DEHP has been shown to cause abnormal estrous cyclicity and accelerate primordial follicle recruitment in female CD-1 mice [45]. The 500 mg/kg/day dose of DEHP was selected because it has been shown to cause abnormalities in spermatagonial stem cells across multiple generations in male CD-1 mice [52]. The 750 mg/kg/day dose of DEHP was selected because adult exposure has been shown to cause abnormal estrous cyclicity and accelerate primordial follicle recruitment in adult female CD-1 mice [45].

Animals and dosing paradigm

Adult female and male CD-1 mice (Charles River, USA) were housed at 25°C in conventional polysulfone, ventilated cages on 12L:12D cycles. The mice were fed Teklad Rodent Diet 8604 (Harlan) and provided highly purified water (reverse osmosis filtered water) in polysulfone water bottles *ad libitum*. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and abide by the guidelines set forth by the National Institute of Health for the Care and Use of Laboratory Animals.

At 8 weeks of age, female mice (F0) were mated with control male mice of the same age. The female mice were monitored twice a day for the presence of a copulatory vaginal sperm plug to confirm mating. Once a copulatory vaginal sperm plug was confirmed, the presence of which was considered gestational day (GD) 0.5, the females were removed, weighed, and individually housed. Subsequently, the mice were weighed twice a week to confirm successful pregnancy. From GD 10.5 until birth of the pups, pregnant dams (F0) were orally dosed once a day with the vehicle control (tocopherol-stripped corn oil) or with DEHP (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, 750 mg/kg/day) by placing a pipette tip with the dosing solution into the cheek pouch of the mouse. This dosing regimen was selected to mimic oral exposure to DEHP in humans [1, 45, 51]. The doses were calculated and adjusted based on daily body weights, and delivered in 25 – 33 µL of tocopherol-stripped corn oil. The treatment window was chosen because it is a critical time period of ovarian development. Specifically, this is when primordial germ cells arrive at the gonad [55, 56], sex determination occurs [57], and global demethylation and imprint erasure of primordial germ cells occur [58].

Pregnant mice were allowed to deliver naturally and the day of birth was considered postnatal day (PND) 0. Mice born from the F0 generation were labeled the F1 generation.

Female mice from the F1 generation were mated with non-treated male CD-1 mice to produce the F2 generation. Females from the F2 generation were mated with non-treated male CD-1 mice to produce the F3 generation. No mice were mated with family members. At PND 21, mice were euthanized by CO_2 affiliation followed by cervical dislocation. PND 21 was selected because mice are juvenile and not sexually mature. Further, sex steroid hormones are not actively produced in the ovary and no corpora lutea are present. Whole ovaries were collected from each mouse. One ovary was fixed in Dietrich's fixative for immunohistochemistry and the second ovary was immediately frozen in liquid nitrogen and stored at -80C for RNA and DNA extraction.

RNA sequencing analysis

Frozen whole ovaries collected at PND 21 from control and 20 µg/kg/day (n = 3 ovaries/treatment group) from the F3 generation were used for RNA sequencing. Raw reads were checked for quality using FASTQC (v 0.11.5) then trimmed and filtered using Trimmomatic (v 0.36) to remove residual adapter content, low quality bases (Phred quality score < 28), and resulting reads shorter than 30 nt. Trimmed/filtered reads were aligned to NCBI's Mus musculus GRCm38.p6 genome and gene model annotation release 106 using STAR (v 2.5.3a). Post-alignment gene counts were then determined for each NCBI EntrezGene ID using featureCounts from Subread (v 1.5.2-pl) with multi-mapping reads excluded.

The raw read counts were input into R [59] (v 3.4.3) for pre-processing and analysis together using Bioconductor [60] packages as listed below. Approximately ~23 million reads aligned uniquely within the 41,595 *M. musculus* genes. We used TMM method [61] in the edgeR

package [62] (v 3.20.5) to normalize the counts to log2-transformed counts per million (logCPM), using the cpm() function with prior.count = 3. Specifically, 25,141 genes did not have logCPM > log2(0.5) in at least three samples and were filtered out, leaving 16,454 genes to be analyzed for differential expression. TMM-values and logCPM normalized values were re-calculated with prior.count = 3 after gene filtering. Principle components analysis clustering of the samples (Data not shown) indicated one of the treatment replicates was more variable than the other two. Rather than remove this sample completely, we did a surrogate variables analysis [63, 64] on the logCPM values, which estimated one surrogate variable that corrected for the difference in this replicate (data not shown). This surrogate variable was added to edgeR's quasi-likelihood negative binomial generalized log-linear model [65], which was fit on the original read counts + TMM values to find differential expression between the treated and control groups. Multiple hypothesis test correction was done using the False Discovery Rate method [66].

Data obtained from RNA sequencing were functionally analyzed using The Database of Annotation, Visualization, and Integrated Discovery Bioinformatics (DAVID) 6.8 following the previously published protocol [67, 68]. Genes with a false discovery rate < 0.62 and $p < 0.007$ were entered into DAVID for functional annotation analysis for a total of 177 genes. "Gene_Ontology" and "Pathways" and the denoted DAVID defined defaults were selected for functional annotation clustering. To determine if functional gene groups were valuable, annotation clusters with a significant enrichment score ≥ 1 were further explored [67].

Gene expression analysis

Frozen whole ovaries collected at PND 21 were used for quantitative real-time polymerase chain reaction (qPCR) analysis (n= 3 – 6 ovaries/treatment group). Total RNA (>100 ng) was extracted from the whole ovaries using the AllPrep DNA/RNA Mini Kit (Qiagen, Austin, TX, USA) according to the manufacturer's protocol, including DNase digestion. Total RNA (100 ng) was reverse transcribed to complementary DNA (cDNA) using the iScript RT Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Each cDNA sample was diluted 1:8 using nuclease-free water prior to qPCR analysis. Analysis of qPCR was performed using the CFX96 C1000 Real-Time PCR Detection System and CFX Manager Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Each qPCR reaction was done in duplicates using 2 µL of cDNA, forward and reverse primers (5 pmol) for select genes, nuclease-free water, and SsoFastEvaGreen Supermix for a final reaction volume of 10 µL. Target genes were analyzed in reference to the housekeeping gene, beta-actin (*Bactin*). A list of gene primers (Integrated DNA Technologies, Coralville, IA, USA) and the housekeeping gene, beta-actin, are included in the supplementary files (Table S1).

The CFX96 C1000 Real-Time PCR Detection machine quantifies the amount of PCR product generated by measuring SsoFastEvaGreen dye (Bio-Rad Laboratories, Inc., Hercules, CA) that fluoresces when bound to double-stranded DNA. The qPCR program consisted of an enzyme activation step (95 °C for 1 min), an amplification and quantification program (40 cycles of 95 °C for 10 s, 60 °C for 10 s, single fluorescence reading), a 72 °C for 5 min, a melt curve (65 °C–95 °C heating 0.5 °C/s with continuous fluorescence readings), and a final step at 72 °C for 5 min as per the manufacturer's protocol. All gene expression data were normalized to the

housekeeping gene. Relative fold changes were calculated as the ratio to control group level and were analyzed using a mathematical model for relative quantification of real-time PCR data developed by Pfaffl [69].

DNA methylation analysis

DNA was extracted from frozen whole PND 21 ovaries using the AllPrep DNA/RNA Mini Kit (Qiagen, Austin, TX, USA) per the manufacturer's protocol. DNA was extracted, eluted in 100 µL of EB buffer, and stored in -80 C until further DNA methylation testing. To measure global DNA methylation status, the enzyme-linked immunosorbent assay MethylFlash Methylated DNA 5-mC Quantification Kit (Colorimetric assay, Epigentek Group Inc., Farmingdale, NY, USA) was used according to the manufacturer's protocol. Briefly, ovarian DNA (100 ng) was added to high affinity strip wells. Methylated DNA was detected using capture and detection antibodies for 5-methyl cytosine (5-mC) and quantified by reading absorbance at 450 nm using a 354 Multiskan Ascent Microplate Reader (Thermo Electron Corp., Shanghai, China). The absolute amount and percentage of methylated DNA were calculated using the absolute quantification method per the manufacturer's protocol. Briefly, a standard curve was calculated from five known concentrations of methylated DNA (0.5, 1, 2, 5, 10 ng). The slope of the standard curve was quantified and used in the provided formulas per the manufacturer's protocol to calculate the amount and percentage of methylated DNA (5-mC) in each sample.

Statistical analyses

Data were expressed as the mean ± standard error of the mean (SEM). In all generations, data from multiple female pups originating from the same litter were averaged and combined as n = 1, and data from at least 3 separate litters were used in the analyses. Data were analyzed by comparing treatment groups to control using IBM SPSS version 24 software (SPSS Inc., Chicago, IL, USA). Outliers were removed by the Grubb's test using GraphPad outlier calculator software (GraphPad Software Inc., La Jolla, CA, USA). Data that were continuous were assessed for normal distribution by Shapiro-Wilk analysis. If data met assumptions of normal distribution and homogeneity of variance, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey HSD or Dunnett 2-sided *post-hoc* comparisons. However, if data met assumptions of normal distributions, but not homogeneity of variance, data were analyzed by ANOVA followed by Games-Howell or Dunnett's T3 *post-hoc* comparisons. If data were presented as percentages or were not normally distributed, the independent sample Kruskal-Wallis H followed by Mann-Whitney U non-parametric tests were performed. For all comparisons, statistical significance was determined by a p-value ≤ 0.05. In instances in which p-values were greater than 0.05, but less than 0.10, data were considered to exhibit a trend towards significance.

5.4 Results

The effects of ancestral exposure to DEHP on gene expression in the F3 generation as determined by RNA sequencing and The Database of Annotation, Visualization, and Integrated Discovery Bioinformatics

Functional annotation gene clustering analysis via DAVID provided 5 annotation clusters from the "Gene_Ontology" selection (Figure 5.1A). Within the annotation cluster containing the highest enrichment score was insulin-like growth factor binding, regulation of cell growth, and growth factor binding (Figure 5.1A). Functional annotation gene clustering from the "Pathway" selection provided 1 annotation cluster (Figure 5.1B). Within the annotation cluster, extra cellular matrix-receptor interaction, amoebiasis, focal adhesion, and the PI3K-Akt signaling pathway were listed (Figure 5.1B). Based on these results, subsequent qPCR was conducted to assess the effects of DEHP exposure on ovarian gene expression.

The effects of DEHP exposure on hormone receptors and insulin-like growth factor gene expression in ovaries from the F1 – F3 generations

Several studies suggest that DEHP and its many metabolites act through steroid hormone receptors and peroxisome proliferator-activated receptors (PPAR) [70, 71]. Therefore, the current study examined the effects of prenatal and ancestral exposure to DEHP on the expression of hormone receptors and PPARs. Further, based on the RNA sequencing results, the insulin-like growth factor (IGF) family was examined. In the F1 generation, prenatal exposure to DEHP did not affect the expression of *Ppara*, *Fshr*, or *Igfbp4* compared to controls (Figures 5.2A, 5.2E, and 5.2G). However prenatal exposure to DEHP decreased the expression of *Pparg* in the 20

µg/kg/day and 750 mg/kg/day groups, increased *Esr2* expression in the 750 mg/kg/day group, decreased the expression of *Ar* in the 20 µg/kg/day group, and increased the expression of *Igf1* in the 500 mg/kg/day group compared to controls (Figures 5.2B, 5.2C, 5.2D, and 5.2F, n = 3 – 5 ovaries/treatment group, $p \leq 0.05$). In the F2 generation, exposure to DEHP did not affect the expression of *Ppara, Pparg, Ar, Fshr,* or *Igf1* compared to controls (Figures 5.2A, 5.2B, 5.2D, 5.2E, and 5.2F). In contrast, exposure to DEHP decreased the expression of *Esr2* in the 20 µg/kg/day group and decreased *Igfbp4* expression in the 500 mg/kg/day group compared to controls (Figures 5.2C and 5.2G, n = 3 ovaries/treatment group, $p \leq 0.05$). In the F3 generation, ancestral exposure to DEHP did not affect the expression of *Ppara, Pparg, Fshr, Igf1,* or *Igfbp4* compared to controls (Figures 5.2A, 5.2B, 5.2E, 5.2F, and 5.2G). However ancestral exposure to DEHP decreased the expression of *Esr2* in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups, and decreased the expression of *Ar* in the 200 µg/kg/day group compared to controls (Figures 5.2C and 5.2D, n = 3 – 6 ovaries/treatment group, $p \leq 0.05$ but $p = 0.071$ for *Ar* in 200 µg/kg/day).

The effects of DEHP on steroidogenic enzyme gene expression in ovaries from the F1 – F3 generations

Our previous work showed that prenatal DEHP exposure dysregulated steroid hormone levels in F2 generations, but not the F1 and F3 generations of mice at PND 21 [25]. The current work was performed to examine estrogen synthesis and expand our knowledge of DEHP dysregulation of steroid hormones by determining if it is due to prenatal or ancestral DEHP effects on expression of steroidogenic enzymes. In the F1 generation, prenatal exposure to DEHP did not affect the expression of *Star, Hsd17b1, Cyp11a1, Cyp17a1, Cyp19a1, Cyp1a1,* or

Cyp1b1 (Figures 5.3A, 5.3C, 5.3D, 5.3E, 5.3F, 5.3G, and 5.3H), but DEHP at 750 mg/kg/day increased the expression of *Hsd3b1* compared to controls (Figure 5.3B, n = 3 – 5 ovaries/treatment group, $p \leq 0.05$). In the F2 generation, exposure to DEHP decreased the expression of *Star* in the 20 µg/kg/day group, decreased *Hsd3b1* expression in the 20 µg/kg/day group, decreased *Hsd17b1* expression in the 20 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day groups, and decreased *Cyp19a1* expression in the 20 µg/kg/day and 500 mg/kg/day groups compared to controls (Figures 5.3A, 5.3B, 5.3C, and 5.3F, n = 3 ovaries/treatment group, $p \leq 0.05$, but $p = 0.081$ for *Star* in 20 µg/kg/day). In the F3 generation, ancestral exposure to DEHP did not affect the expression of *Star, Hsd3b1, Cyp11a1, Cyp17a1, Cyp19a1,* or *Cyp1a1* compared to controls (Figures 5.3A, 5.3B, 5.3D, 5.3E, 5.3F, and 5.3G), but ancestral exposure decreased the expression of *Hsd17b1* in the 20 µg/kg/day and 750 mg/kg/day groups and decreased *Cyp1b1* expression in the 200 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day treatment groups compared to controls (Figures 5.3C and 5.3H, n = 3 – 6 ovaries/treatment group, $p \leq 0.05$, but $p = 0.068$ and 0.069 for *Cyp1b1* in 500 mg and 750 mg/kg/day, respectfully).

The effects of DEHP exposure on phosphoinositide 3-kinase pathway gene expression ovaries from the F1 – F3 generations

Our previous work showed that ancestral exposure to DEHP accelerated primordial follicle recruitment in the F3 generation, but not in the F1 and F2 generations of mice [25]. Previous studies also indicate that adult exposure to DEHP dysregulates the PI3K pathway, a critical pathway for primordial follicle recruitment [45]. Thus, we examined the effects of prenatal and ancestral exposure to DEHP on the PI3K factors in the F1 – F3 generations. In the

F1 generation, prenatal exposure to DEHP did not affect the expression of *Foxl2, Kitl, Mtorc1, Foxo3a, Pdk1, Kit, Tsc1,* or *Rps6* compared to controls (Figures 5.4A, 5.4B, 5.4C, 5.4D, 5.4F, 5.4G, 5.4H, and 5.4J). However, prenatal exposure to 20 µg/kg/day of DEHP increased the expression of *Pten* compared to controls (Figure 5.4E, n = 3 – 5 ovaries/treatment group, $p \leq 0.05$). In the F2 generation, exposure to DEHP did not affect the expression of *Foxl2, Kitl, Pdk1, Kit, Tsc1,* or *Rps6* compared to controls (Figures 5.4A, 5.4B, 5.4F, 5.4G, 5.4H, and 5.4J), but DEHP exposure increased the expression of *Mtorc1* at 20 µg/kg/day and 750 mg/kg/day and decreased *Mtorc1* expression at 200 µg/kg/day, decreased the expression of *Foxo3a* in the 20 µg/kg/day group and increased *Foxo3a* expression in the 750 mg/kg/day group, and decreased the expression of *Pten* in the 20 µg/kg/day group compared to controls (Figures 5.4C, 5.4D, and 5.4E, n = 3 ovaries/treatment group, $p \leq 0.05$). In the F3 generation, ancestral exposure to DEHP did not affect the expression of *Foxl2, Kitl, Mtorc1, Foxo3a, Kit,* or *Tsc1* compared to controls (Figures 5.4A, 5.4B, 5.4C, 5.4D, 5.4G, and 5.4H). Ancestral exposure to DEHP decreased the expression of *Pten* in all treatment groups, decreased *Pdk1* expression in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups, and decreased *Rps6* expression in the 20 µg/kg/day group compared to controls (Figures 5.4E, 5.4F, and 5.4J, n = 3 – 6 ovaries/treatment group, $p \leq 0.05$).

The effects of DEHP exposure on cell cycle regulator gene expression in ovaries from the F1 – F3 generations

Our previous work showed the prenatal exposure to DEHP dysregulated folliculogenesis at PND 21 in all three generations of mice [25]. Folliculogenesis is regulated by many factors, but the cell cycle regulators are heavily involved in cell proliferation and follicle growth [49, 72]. Therefore, we measured the mRNA expression levels of cyclins, cyclin dependent kinases, and

cyclin dependent kinase inhibitors. In the F1 generation, prenatal exposure to DEHP increased the expression of *Ccna2* in the 500 mg/kg/day group, decreased the expression of *Ccnd2* in the 750 mg/kg/day group, decreased the expression of *Cdkn1a* in the 750 mg/kg/day group, increased the expression of *Cdkn1c* in the 200 µg/kg/day group, and increased the expression of *Cdkn2a* expression in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups compared to controls (Figure 5.5A, 5.5C, 5.5F, and 5.5H n = 3 – 6 ovaries/treatment group, $p \leq 0.05$, but p = 0.076 for *Ccna2* in 500 mg/kg/day, p = 0.101 for *Ccnd2* in 20 µg/kg/day, and p = 0.055 for *Cdkn2a* for 750 mg/kg/day). In the F1 generation, prenatal exposure to DEHP did not affect the expression of *Ccnb1*, *Ccne1, Cdk4,* or *Cdkn1c* compared to controls (Figures 5.5B, 5.5E, 5.5D, and 5.5G). In the F2 generation, exposure to DEHP did not affect the expression of *Ccna2, Ccnd2, Ccne1, Cdk4, Cdkn1a, Cdkn1c,* or *Cdkn2a* compared to controls (Figures 5.5A, 5.5C, 5.5D, 5.5E, 5.5F, 5.5G, and 5.5H). However, exposure to DEHP at 500 mg/kg/day decreased the expression of *Ccnb1* compared to controls, but it was borderline statistically significant (Figure 5.5B, n = 3 ovaries/treatment group, p = 0.057). In the F3 generation, ancestral exposure to DEHP decreased the expression of *Ccna2* in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups, decreased *Ccnb1* expression in the 750 mg/kg/day group, decreased *Ccnd2* expression in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups, decreased *Cdk4* expression in the 750 mg/kg/day group, and decreased the expression of *Cdkn2a* in the 20 µg/kg/day and 200 µg/kg/day groups compared to controls (Figures 5.5A, 5.5B, 5.5C, 5.5E, and 5.5H n = 3 – 6 ovaries/treatment group, $p \leq 0.05$, but p = 0.084 and 0.060 for *Ccnd2* in 200 µg/kg/day and 750 mg/kg/day, respectfully). Further, in the F3 generation, ancestral exposure to DEHP did not affect the expression of *Ccne1, Dckn1a,* or *Cdkn1c* compared to controls (Figures 5.5D, 5.5F, and 5.5G).

The effects of DEHP exposure on apoptosis and oxidative stress pathway gene expression in ovaries from the F1 – F3 generations

Our previous study showed the prenatal exposure to DEHP decreased the percentage of atretic follicles in the F1 generation [25]. The B-cell lymphomas/leukemia-2 (Bcl-2) family includes inhibitors and promoters of apoptosis in the ovary [47]. The balance of promotors and inhibitors of apoptosis is critical for the healthy development and maintenance follicular cells. The Bcl-2 family has been shown to directly regulate apoptosis in the ovary [47, 73]. In addition, oxidative stress is an imbalance of pro-oxidant molecules and anti-oxidant defenses and the balance of these factors are critical for adequate growth and development of follicles [48]. Thus, we examined the effects of prenatal and ancestral exposure to DEHP on the expression of the Bcl-2 family and oxidative stress factors in the ovary of the F1 – F3 generations.

In the F1 generation, prenatal exposure to DEHP did not affect the expression of *Bcl2*, *Bax, Bad, Casp3, Casp8, Catalase, Gpx,* or *Gsr* compared to controls (Figures 5.6A, 5.6B, 5.6C, 5.6E, 5.6F, 5.6H, 5.6J, and 5.6K). In contrast, prenatal exposure to DEHP at 750 mg/kg/day decreased the ratio of *Bax*/*Bcl2* and DEHP at 500 mg/kg/day increased the expression of *Bok* compared to controls, but it was borderline statistically significant (Figure 5.6D and 5.6G, n = 3 – 5 ovaries/treatment group, $p \leq 0.05$ and $p = 0.076$ for *Bok*). In the F2 generation, exposure to DEHP did not affect the expression of *Bcl2, Bax, Bax*/*Bcl2* ratio, *Bok, Catalase,* or *Gsr* compared to controls (Figures 5.6A, 5.6B, 5.6D, 5.6E, 5.6H, and 5.6K). However, exposure to 20 µg/kg/day of DEHP decreased the expression of *Bad, Casp3,* and *Casp8,* and exposure to 500 mg/kg/day of DEHP increased the expression of *Gpx* compared to controls (Figures 5.6C, 5.6E, 5.6F, and 5.6J, n = 3 ovaries/treatment group, $p \leq 0.05$). In the F3 generation, ancestral exposure to DEHP decreased the expression of *Bcl2* in all treatment groups, increased the expression of

Bax/*Bcl2* ratio in the 20 µg/kg/day, 200 µg/kg/day, and 500 mg/kg/day groups, decreased the expression of *Casp3* in the 20 µg/kg/day, 200 µg/kg/day, and 500 mg/kg/day groups, decreased the expression of *Casp8* in all treatment groups, decreased the expression of *Bok* in the 20 µg/kg/day group, decreased the expression of *Gpx* in the 750 mg/kg/day group, and decreased the expression of *Gsr* in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups compared to controls (Figures 5.6A, 5.6D, 5.6E, 5.6F, 5.6G, 5.6J, and 5.6K, n = 3 – 6 ovaries/treatment group, $p \leq 0.05$, but $p = 0.071$ for *Casp8* in 750 mg/kg/day, $p = 0.088$ for *Bok* in 20 µg/kg/day, and $p = 0.067$ for *Gsr* in 200 µg/kg/day).

The effects of DEHP exposure on DNA methyltransferases and ten-eleven translocation enzyme gene expression in ovaries from the F1 – F3 generations

Although previous studies demonstrate that DEHP exposure causes transgenerational inheritance of ovarian dysfunction [25, 74, 75], the DNA methylation mediators underlying these changes have not been well studied. Therefore, we examined the expression levels of DNA methyltransferases (DNMT) and ten-eleven translocation enzyme (TET) gene expression in the F1 – F3 generations. In the F1 generation, prenatal exposure to DEHP did not affect the expression of *Dnmt3a, Dnmt3b, Tet1, Tet2,* and *Tet* compared to controls (Figures 5.7B, 5.7C, 5.7D, 5.7E, and 5.7F). However, prenatal exposure to DEHP at 750 mg/kg/day increased the expression of *Dnmt1* compared to controls, but it was borderline statistically significant (Figure 5.7A, n = 3 – 5 ovaries/treatment group, $p = 0.068$). In the F2 generation, exposure to DEHP did not affect the expression of *Dnmt1, Dnmt3a,* or *Dnmt3b* compared to controls (Figures 5.7A, 5.7B, and 5.7C). In contrast, DEHP exposure decreased the expression of *Tet1* in all groups, decreased the expression of *Tet2* in the 20 µg/kg/day group, and decreased the expression of *Tet3*

in the 500 mg/kg/day group compared to control, but it was borderline statistically significant (Figures 5.7D, 5.7E, and 5.7F, n = 3 ovaries/treatment group, $p \leq 0.05$, but $p = 0.085$ for *Tet3* in 500 mg/kg/day). In the F3 generation, ancestral exposure to DEHP decreased the expression of *Dnmt1* in all groups compared to control, but some of the decreases were borderline statistically significant (Figure 5.7A, n = 3 – 6 ovaries/treatment group, $p \leq 0.05$, but $p = 0.097$ for 200 µg/kg/day, $p = 0.095$ for 500 mg/kg/day, and $p = 0.059$ for 750 mg/kg/day). Further, ancestral exposure to DEHP decreased the expression of *Dnmt3a* in the 750 mg/kg/day group, decreased the expression of *Dnmt3b* in all groups, decreased *Tet2* expression in the 200 µg/kg/day and 750 mg/kg/day groups, and decreased *Tet3* expression in the 200 µg/kg/day and 750 mg/kg/day groups compared to controls (Figures 5.7B, 5.7C, 5.7E, and 5.7F, n = 3 – 6 ovaries/treatment group, $p \leq 0.05$, but $p = 0.064$ for *Tet2* in 750 mg/kg/day and $p = 0.063$ for *Tet3* in 200 µg/kg/day). In the F3 generation, ancestral exposure to DEHP did not affect the expression of *Tet1* compared to controls (Figure 5.7D).

The effects of DEHP exposure on DNA methylation percentage in whole ovaries from the F1 – F3 generations

Previous studies determined that prenatal exposure to DEHP causes both multigenerational and transgenerational inheritance in ovarian dysfunction [23-25]. Transgenerational inheritance is thought to be mediated by epigenetic mechanisms, and DNA methylation is a commonly studied epigenetic mechanism. Therefore, the current study measured the percentage of 5-mC in the whole ovary in the F1 – F3 generations. In the F1 generation, prenatal exposure to 20 µg/kg/day of DEHP increased the percentage of 5-mC compared to controls and in the F3 generation, ancestral exposure to 500 mg/kg/day and 750 mg/kg/day of

DEHP decreased the percentage of 5-mC in the whole ovary compared to controls (Figure 5.8, n = 3 – 7 ovaries/treatment group, $p \leq 0.05$).

5.5 Discussion

Our previous study showed that prenatal and ancestral exposure to DEHP disrupted sex steroid hormone levels in the F1 and F2 generations, disrupted ovarian follicle counts in the F1 – F3 generations, and altered select reproductive outcomes in the F1 – F3 generations [24, 25]. Our current study provides additional information on the multigenerational and transgenerational effects of DEHP exposure on the ovary. We show that prenatal exposure to DEHP disrupts the expression of the sex steroid hormone synthesis pathway, factors in the PI3K pathway, steroid hormone receptors, DNA demethylation processes, and DNA methylation in the F1 and the F2 generations of the ovary. Further, we show that ancestral exposure to DEHP disrupts the expression of estrogen metabolism, PI3K pathway, cell cycle regulators, apoptosis and oxidative stress factors, estrogen receptor beta, DNA methylation and demethylation factors, and DNA methylation in the F3 ovary. This study provides potential mechanisms and pathways explaining how both prenatal and ancestral exposure to DEHP disrupt ovarian functions in the F1 – F3 generations of mice.

In this study, mice were orally exposed daily to DEHP starting at embryonic day 10.5 and ending at birth. During this exposure window, primordial germ cells in the fetus (F2 generation) migrate to the genital ridge and undergo mitosis, meiosis, methylation, and demethylation processes [76-79]. We anticipate that this window of exposure targets epigenetic inheritance and likely causes transgenerational phenomenon in the F3 generation [79]. This window of exposure

is critical because the F1, F2, and F3 generations receive exposure at different developmental windows. The F1 generation is exposed as a developing pup, and therefore, the hypothalamus-pituitary-gonadal axis is directly exposed to DEHP. The F2 generation is exposed as the developing germ cells in the gonad. The F3 generation is not directly exposed to DEHP and thus, it is the first generation to experience transgenerational inheritance. Each generation is at different developmental time points, therefore, we anticipated that the effects of DEHP on ovarian functions would be different in each generation.

Our results indicate that DEHP exposure altered the expression of sex steroid hormone receptors in the F1, F2, and F3 generations. DEHP is a known endocrine disrupting chemical with studies showing that it acts through PPAR, estrogen receptors, and the androgen receptor [70, 80, 81]. Our study showed that in the F1 generation, prenatal DEHP exposure decreased the expression of *Pparg* and *Ar*, but increased the expression of *Esr2*. In the F2 generation, DEHP exposure decreased the expression of *Esr2*. In the F3 generation, ancestral DEHP exposure decreased the expression of *Esr2* and *Ar*. A previous study demonstrated that direct DEHP inhibited ERα, ERβ, and AR and that DEHP metabolites activated PPARα and PPARγ [70]. However, in our study, DEHP exposure only affected the expression of *Pparg, Ar,* and *Esr2* in the F1 generation. The other study was performed *in vitro* and our current study was performed *in vivo* and therefore, it is likely that the experimental set up contributes to the differences in receptor activation. A different study demonstrated that DEHP exposure represses *Esr1* gene expression via PPARα-dependent pathways in a multigenerational manner [80]. However, in our study, we did not observe a significant change in *Ppara* gene expression in any generation. It was surprising that we did not see changes in *Ppar* expression in the F3 generation because the

endocrine disrupting effects of DEHP are thought to be mediated through PPAR action [80, 82-84].

Our study showed that DEHP exposure increased the expression of *Igf1* in the F1 generation and decreased the expression of *Igfbp4* in the F2 generation, but did not affect the gene expression in the F3 generation. Our data indicate that prenatal DEHP exposure caused multigenerational changes on gene expression on insulin-like growth factor (*Igf*). Mice lacking IGF1 are infertile with follicles arrested in the preantral stage, suggesting that it is important for follicular development and proliferation and differentiation of granulosa cells [85]. Therefore, an increase in *Igf1* expression suggests that ovaries would have contained mature follicle types. However, we observed the opposite in the F1 generation; we observed that DEHP exposure decreased antral follicle numbers [25]. It is likely that the increased *Igf1* expression by DEHP exposure is a compensatory mechanism to encourage the growth of mature follicle types in the ovary.

The insulin-like growth factor binding proteins (IGFBPs) are necessary to transport IGFs into the bloodstream and ultimately determine IGF bioavailability [85]. Therefore, a decrease in *Igfbp4* expression may induce a decrease in bioavailability of IGFs. Further, *Igfbp4* expression is restricted to apoptotic and atretic follicles [47]. Interestingly, in our previous study, we did not observe changes in atretic follicle numbers [25] even though we observed decreased *Igfbp4* expression.

Our results show that DEHP exposure disrupted steroidogenesis in the F2 and F3 generations, but not in the F1 generation. According to our previous study, prenatal DEHP exposure did not affect serum 17β-estradiol levels in the F1 generation at PND 21 [25]. Therefore, it is not surprising that prenatal DEHP exposure did not significantly affect the

expression of sex steroid hormone synthesis enzymes in the F1 generation. However, in the F2 generation, our previous study showed that prenatal exposure to DEHP borderline decreased serum 17β-estradiol levels and increased serum progesterone levels in the 20 µg/kg/day treatment group compared to control [25]. In our current study, DEHP exposure at 20 µg/kg/day decreased the expression of *Star*, *Hsd3b1*, *Hsd17b1*, and *Cyp19a1* in the F2 generation. This decrease in enzymes correlates well with the previously observed serum sex steroid hormone levels. Likely, the increase in serum progesterone level is due to the decrease of enzymes necessary to further biotransform it to androgens and estrogens. Further, the decrease of *Hsd17b1* and *Cyp19a1* likely leads to a decrease in serum 17β-estradiol levels because these two enzymes biotransform estrone and testosterone into 17β-estradiol, respectfully [86]. Finally, in the F3 generation, we observed a decrease with DEHP exposure in *Hsd17b1* expression, which is important for biotransforming androstenedione into testosterone and estrone into 17β-estradiol [86]. However, in our previous study, we did not observe a serum sex steroid hormone change [25], but we observed a DEHP-induced decrease in *Cyp1b1*, an enzyme important for hydroxylation of 17β-estradiol [87, 88]. It may be that the decreases in *Hsd17b1* and *Cyp1b1* are compensatory mechanisms in the ovary to keep serum 17β-estradiol levels at a normal and healthy level.

Interestingly, our results are in contrast with another study that exposed mice to DEHP during an early developmental time period. Specifically, Pocar *et al.* perinatally dosed mice throughout gestation and lactation with low doses of DEHP and observed decreases in steroidogenic enzyme expression in the F1 generation and not the F2 or F3 generations [75]. The reason why our results and Pocar *et al.* vary may be due to the many differences between the experiments. In our study, we dosed animals only during the second half of gestation and our

doses varied (20 µg/kg/day – 750 mg/kg/day), whereas Pocar *et al.* dosed animals throughout lactation and gestation with different doses (50 µg/kg/day and 5 mg/kg/day) [75]. Due to DEHP and its endocrine disrupting activities, the timing and dose of exposure greatly influences the observed effects [79, 89].

Results from out study indicate that prenatal and ancestral DEHP exposure disrupted the PI3K pathway in the F1, F2, and F3 generations. In the F1 generation, prenatal DEHP exposure increased *Pten* expression in the PI3K pathway but it did not affect other factors in the pathway. *Pten* is a gene that encodes the PI3K negative regulator; if deleted, the entire pool of primordial follicles activate [42]. Therefore, an increase in *Pten* expression suggests a primordial follicle quiescence. Interestingly, in our previous study, we observed data supporting primordial follicle quiescence. At PND 21, prenatal DEHP exposure decreased the number of antral follicles in the F1 generation, suggesting primordial follicle quiescence [25, 90]. Additionally, at PND 60, prenatal DEHP exposure decreased the number of primary and preantral follicle numbers further suggesting that primordial follicle activation may be impeded in the F1 generation, though we did not measure gene expression at this time point [25]. In our study, the increased *Pten* expression supports follicle count numbers observed in our previous study in the F1 generation [25]. These data are similar to a study by Hannon *et al.* in which mice were exposed to DEHP during adulthood and the expression of *Pten* was measured in whole ovaries. Hannon *et al.* found that DEHP exposure decreased the expression of *Pten* in mice exposed to DEHP [45]. Although our study and Hannon *et al.* did not use the same exposure window, it is interesting that DEHP exposure has a strong effect on *Pten* expression. In the F2 generation, exposure to DEHP altered the expression of *Mtorc1* and *Foxo3a*, but decreased the expression of *Pten* compared to control. Similar to *Pten*, *Foxo3a* is responsible for primordial follicle quiescence and a lack of *Foxo3a*

promotes primordial follicle activation [91]. In contrast, *Mtorc1* is involved in primordial follicle activation and regulates protein synthesis and cell growth, whereas a decrease in *Mtorc1* expression promotes primordial follicle quiescence [90, 92]. In the F3 generation, ancestral exposure to DEHP decreased the expression of *Pten, Pdk1,* and *Rps6*. PI3K signaling is mediated through *Pdk1* and it signals to activate AKT to maintain primordial follicles survival [93]. Further, *Rps6* encodes for a ribosomal protein necessary to maintain the survival of primordial follicles in relation to PDK1-AKT signaling [93]. Our previous data showed that at PND 21, ancestral DEHP exposure decreased primordial follicle numbers [25]. A correlated decrease in *Pten, Pdk1,* and *Rps6* expression correlates well with decreased primordial follicle numbers because decreased *Pten* expression suggests that primordial follicles activate and continue folliculogenesis [42]. Further, decreased *Pdk1* and *Rps6* expression decreases primordial follicle survival [93]. Therefore, decreased expression of these factors supports follicle count numbers in the F3 generation and provides a potential mechanism for follicle count disruption at PND 21.

Prenatal and ancestral exposure to DEHP significantly decreased gene expression of cell cycle regulators in the F1 and F3 generations. In somatic cells, the cell cycle is made of four phases with different cyclin-dependent kinases and cyclins to control the cell cycle [94]. Cyclin A2 is expressed during the S phase and is critical for DNA replication [49]. Cyclin B1 is necessary for cell cycle progression through mitosis [49]. Cyclin D2 binding to CDK4 is a critical positive regulator for ovarian granulosa cell proliferation in response to follicle-stimulating hormone (FSH) [72, 95]. In the F1 generation, DEHP exposure increased *Ccna2, Cdkn1c,* and *Cdkn2a* but decreased *Ccnd2* and *Cdkn1a* expression. It is likely that prenatal DEHP exposure inhibits the cell cycle progression by decreasing promotors of the cell cycle such as *Ccnd2* and increasing the expression of inhibitors of cell cycle such as *Cdkn2a* and *Cdkn1c*

[96, 97]. Although the expression of *Cdkn1a*, another cell cycle inhibitor, was decreased in the F1 generation, it is likely that it was not biologically significant enough to counteract the expression of the other inhibitors. The cell cycle changes in the F1 generation support previous data that prenatal DEHP exposure decreases the number of antral follicles at PND 21. Further, the effects of prenatal DEHP exposure on cell cycle regulators is somewhat similar to studies that directly exposed the ovary to DEHP. Direct exposure to DEHP *in vitro* increased the expression of *Ccna2, Ccnb1, Ccnd2, Cdk4,* and *Ccne1* after 72 hours of exposure in the antral follicle [50]. Both experimental models observed increased *Ccna2* expression, however, the two experimental models are vastly different because of the route of exposure (direct *in vitro* vs. prenatal *in vivo*). In the F3 generation, ancestral DEHP exposure decreased the expression of *Ccna2, Ccnb1, Ccnd2,* and *Cdk4*. Decreases in these cyclins and cyclin-dependent kinase suggest that ancestral DEHP exposure reduces cell cycle progression and proliferation and likely leaves these cells in cell cycle arrest. Specifically, *Ccna2, Ccnb1, Ccnd2,* and *Cdk4* encode for cyclin A2, cyclin B1, cyclin D2, and Cdk4 and decreases in these factors decrease the progression of the G_1 phase, S phase, G_2 phase, and they decrease transition from G_2 to M phase [49, 72, 98]. Our study observed significant increases in cell cycle inhibitors in the F1 generation and significant decreases in cell cycle regulation in the F3 generation showing that prenatal DEHP exposure causes transgenerational changes in gene expression.

Our data indicate that DEHP exposure decreased the expression of apoptosis factors in the F2 and F3 generations, but not the F1 generation. Apoptosis is mediated by a balance of pro-apoptotic factors and anti-apoptotic factors [47]. Specifically, *Bad* is a pro-apoptotic factor that utilizes caspases proteases [99]. Some caspases include initiator caspases (*Casp8*) and effector caspases (*Casp3*) that cleave enzymes and proteins necessary for cell viability [47]. In the F2

generation, exposure to DEHP at 20 µg/kg/day decreased the expression of *Bad*, *Casp3*, and *Casp8*. Interestingly, decreases in these genes suggest that DEHP exposure decreases pro-apoptotic factors and favors cell viability without significantly affecting anti-apoptotic factors. In the F3 generation, ancestral DEHP exposure decreases the expression of *Bcl2*, an anti-apoptotic factor [47], decreases the expression of *Bok*, a pro-apoptotic factor [47], and decreases the expression of both *Casp3* and *Casp8*. Although ancestral exposure decreases both pro- and anti-apoptotic factors, it is the ratio of the two that drives either cell survival or death [47]. In the F3 generation, ancestral DEHP exposure increased the ratio of *Bax* to *Bcl2* showing that cells are driven towards an apoptotic fate, however, the expression of both caspases are decreased in the F3 generation. The caspases are cell regulatory enzymes that control cell death [100]. Therefore, the gene expression of the enzymes necessary for cell death is not consistent in our results and is likely that the pro-apoptotic actions are unable to activate the expression of both caspases in the F3 generation.

Our data also indicate that exposure to DEHP did not affect oxidative stress factors in the F1 generation, increased oxidative stress factors in the F2 generation, and decreased oxidative stress factors in the F3 generation. A balance of pro-oxidants and anti-oxidants is necessary for physiological processes, but an imbalance can cause damage to DNA and proteins [48]. A healthy balance is achieved by changing reactive oxygen species, reactive nitrogen species, or anti-oxidant defense mechanism [48, 101]. In our study, in the F2 generation, DEHP exposure increased the expression of *Gpx*, which encodes for an enzyme that degrades hydrogen peroxide into water [48]. In the F3 generation, ancestral DEHP exposure decreased the expression of both *Gpx* and *Gsr*. Although *Gpx* is an anti-oxidant, *Gsr* is necessary to recycle glutathione (GSH) so that it can be recruited to reduce peroxidase [102]. A significant reduction of *Gpx* and *Gsr* may

result in DNA damage and high hydrogen peroxide concentrations, although this current study did not measure DNA damage or reactive oxygen species. However, these data suggest that ancestral DEHP exposure disrupted oxidative stress factors in a transgenerational manner.

Epigenetic modifications are commonly thought to be the mode of action for transgenerational changes. Specifically, we examined DNA methylation mechanisms in all generations. Our data indicate that prenatal exposure to DEHP slightly increased the expression of *Dnmt* enzymes in the F1 generation, but decreased the expression in the F3 generation. Interestingly, we did not observe *Dnmt* expression changes in the F2 generation. Although previous studies demonstrated *Dnmt* expression changes in the F1 generation, no studies to our knowledge have measured *Dnmt* expression changes in the ovary of the F3 generation. One study has perinatally exposed rats to methoxychlor, a well-known endocrine disruptor, and it increased the expression of *Dnmt3b* in PND 50 – 60 ovaries [103]. In another study, rats prenatally exposed to DEHP increased *Dnmt* expression in testicular Leydig cells, increased methylation in promoter regions of steroidogenic transcription factors, and decreased gene expression of steroidogenic enzymes in the F1 generation [104]. This study clearly demonstrated that DNA methylation altered adult testicular function, however, our data do not clearly demonstrate such a connection. Prenatal DEHP exposure slightly increased *Dnmt1* expression, but not significantly, and therefore suggesting that DNA methylation may contributed as a mode of action for altered steroidogenesis and folliculogenesis in the F1 generation at PND 21. Additionally, in the F3 generation, ancestral DEHP exposure decreased *Dnmt* expression, suggesting that DNA methylation plays a major role in regulating gene expression in the ovary.

Next, we measured the gene expression of DNA demethylation contributors in all generations. Our results indicate that prenatal DEHP exposure did not affect the expression of

Tet enzymes in the F1 generation, but DEHP exposure decreased the expression in the F2 generation and ancestral DEHP exposure decreased the expression of *Tet* enzymes in the F3 generation. *Tet* expression correlates to the level of 5-mC because TET oxidized 5-mC into 5-hmC as a demethylation process [32]. In our study, prenatal DEHP exposure increased the percentage of 5mC in the F1 generation and ancestral DEHP exposure decreased the percentage of 5mC in the F3 generation. Although a previous study has shown that overexpression of TET1 reduces genomic 5-mC [105], we did not observe a decrease in *Tet1* expression in the F1 generation that correlates with increased 5-mC in the ovary. Therefore, the increased 5-mC in the F1 generation may not be due to only gene expression of *Tet1* or *Dnmt*, but instead it may be due to changes in protein levels. Interestingly, another study analyzed DNA methylation changes in the rat ovary after prenatal DEHP exposure and found that 406 genes were either hyper- or hypomethylated [106]. Although we did not specifically measure methylation status in genes, our results are similar in that we observed increased 5-mC in prenatally exposed mouse ovaries. In the F2 generation, the percentage of 5-mC was not affected, however, *Tet* expression was decreased with DEHP exposure. The decreased *Tet* expression suggests that DEHP modulates DNA demethylation pathways, but it may not be biologically significant enough to affect 5-mC levels. In the F3 generation, ancestral DEHP exposure decreased *Tet* expression, suggesting that less TET is available to oxidize 5-mC and subsequently, increased 5-mC in the ovary. However, ancestral DEHP exposure decreased the percentage of 5-mC in the ovary. Therefore, *Tet* expression in the ovary may not drive decreased 5-mC. Instead, we anticipate that the decreased *Dnmt3b* expression in the F3 generation results in the decreased 5-mC levels in the ovary. In our study, the changes in 5-mC percentage in the F1 and F3 generations may contribute to some of the gene expression changes observed [40, 107-109], however, additional studies are necessary

to determine if global 5-mC translates to altered methylation in promoters of transcripts for critical ovarian functions. It is critical to selectively measure 5-mC and 5-mhC to adequately determine the roles of *Dnmt*s and *Tet*s in the DNA methylation in the ovary. Without determining the correct percentage of 5-mC and 5-mhC, it is difficult to determine whether the expression levels of *Dnmt*s and *Tet*s are biologically relevant to DNA methylation and demethylation processes.

In summary, our observations indicate that prenatal and ancestral DEHP exposure causes differential gene expression in multiple pathways necessary for healthy ovarian function in the F1, F2, and F3 generations. Further, results from our study suggest that DNA methylation may serve as an epigenetic mechanism, causing some of the transgenerational effects of DEHP. Additional studies are required to understand the developmental epigenetic effects of DEHP and other endocrine disrupting chemicals on ovarian health. In addition, future studies should examine cell types targeted by DEHP exposure. Phthalates, like DEHP, are associated with DNA methylation in cord blood of newborns making studies that examine epigenetic changes extremely important to human medicine [40]. DNA methylation is not the only epigenetic mechanism, thus additional studies should examine other epigenetic mechanisms, such as histone modifications, imprinted genes, and the recently discovered DNA methylation mechanism involving N6-methyladenine [110]. The understanding of epigenetic mechanisms and the understanding of endocrine disruptors on the ovary will help with future pharmaceuticals and devices to combat the toxicant effects on the ovary.

5.6 Table, Figures, and Legends

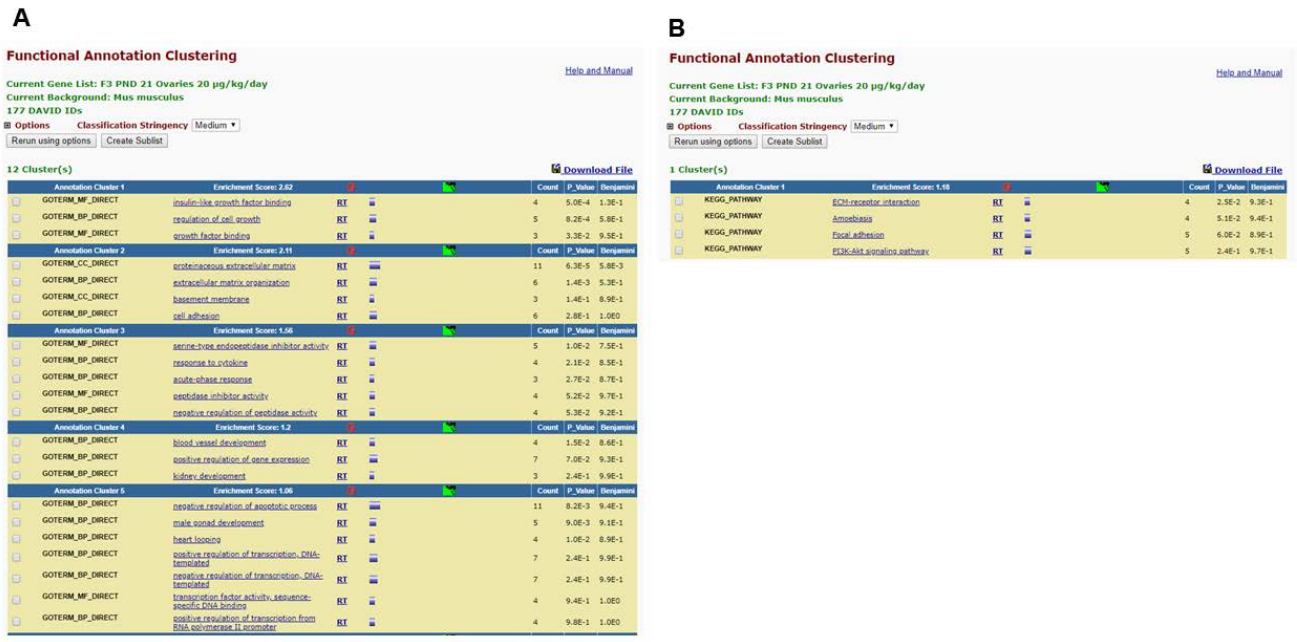
Table 5.1 Sequences of primers sets used for gene expression analysis

Gene name	Symbol	Forward primer	Reverse primer
Actin, beta	*Bactin*	5'-AGCACAGCTTCTTTGCAGCTCCTT-3'	5'-CAGCGCAGCGATATCGTCATCCAT-3'
Steroidogenic acute regulatory protein	*Star*	5'-CAGGGAGAGGTGGCTATGCA-3'	5'-CCGTGTCTTTTCCAATCCTCTG-3'
3β-hydroxysteroid dehydrogenase 1	*Hsd3b1*	5'-CAGGAGAAAGAACTGCAGGAGGTC-3'	5'-GCACACTTGCTTGAACACAGGC-3'
17β-hydroxysteroid dehydrogenase 1	*Hsd17b1*	5'-ACTGTGCCAGCAAGTTTGCG-3'	5'-AAGCGGTTCGTGGAGAAGTAG-3'
Cytochrome P450 11A1	*Cyp11a1*	5'-AGATCCCTTCCCCTGGTGACAATG-3'	5'-CGCATGAGAAGAGTATCGACGCATC-3'
Cytochrome P450 17A1	*Cyp17a1*	5'-CCAGGACCCAAGTGTGTTCT-3'	5'-CCTGATACGAAGCACTTCTCG-3'
Cytochrome P450 aromatase	*Cyp19a1*	5'-CATGGTCCCGGAAACTGTGA-3'	5'-GTAGTAGTTGCAGGCACTTC-3'
Cytochrome P450 1A1	*Cyp1a1*	5'-TGTCAGATGATAAGGTCATCACG-3'	5'-TCTCCAGAATGAAGGCCTCCAG-3'
Cytochrome P450 1B1	*Cyp1b1*	5'-GCGACGATTCCTCCGGGCTG-3'	5'-TGCACGCGGGCCTGAACATC-3'
Forkhead box protein L2	*Foxl2*	5'-GATGGCCAGCTACCCCGAGC-3'	5'-CGCGGGGTCTGGTTTCTCCG-3'
Kit ligand	*Kitl*	5'-AGTGCTTCGCTGTGAACCCTGC-3'	5'-CCAAGCCATGCAAACGGTGCAA-3'
Mammalian target of rapamycin complex 1	*Mtorc1*	5'-GAACCTCAGGGCAAGATGCT-3'	5'-TCTTCAGTCCACTGGCGAAC-3'
Forkhead box O3	*Foxo3a*	5'-AGGAGAGAGCAAGAGCCCAAGC-3'	5'-TCGAACTCTGGGTCCAGCTCCA-3'
Phosphatase and tensin homolog	*Pten*	5'-GCAGAGCAAGCTCAGTGTGGGT-3'	5'-AGGGGGCAAGGTAGGTACGCAT-3'
3-phosphoinositide dependent protein kinase-1	*Pdk1*	5'-AAAAGCAAGCCGGTGGAAAC-3'	5'-CTTGTGAGCATTCCCGCTTG-3'
Mast/stem cell growth factor receptor	*Kit*	5'-AACAACAAAGAGCAAATCCAGGCCC-3'	5'-TGAGCACCATCACAATGATCCCCAT-3'
Tuberous sclerosis 1	*Tsc1*	5'-ATGCTGCCTGTGGGTGGGTGTA-3'	5'-AGGGTGGAACCATGACCAGCCA-3'
Ribosomal protein S6	*Rps6*	5'-GAAGCCAAAGAAAAGCGCCA-3'	5'-TCTCCCCATATTCTAGCAGTCCT-3'
Cyclin A2	*Ccna2*	5'-GCTCTACTGCCCGGAGGCTGA-3'	5'-TGGCCTACATGTCCTCTGGGGAA-3'
Cyclin B1	*Ccnb1*	5'-TGCATTCTCTCAGTGCCCTCCACA-3'	5'-AGACAGGAGTGGCGCCTTGGT-3'
Cyclin D2	*Ccnd2*	5'-CCTTTGACGCAGGCTCCCTTCT-3'	5'-ACCCTGGTGCACGCATGCAAA-3'
Cyclin E1	*Ccne1*	5'-GGTGTCCTCGCTGCTTCTGCTT-3'	5'-CCGGATAACCATGGCGAACGGA-3'
Cyclin-dependent kinase 4	*Cdk4*	5'-AGAAACCCTCGCTGAAGCGGCA-3'	5'-TGGGGGTGAACCTCGTAAGGAGA-3'
Cyclin-dependent kinase inhibitor 1A	*Cdkn1a*	5'-TTAGGCAGCTCCAGTGGCAACC-3'	5'-ACCCCCACCACCACACACCATA-3'
Cyclin-dependent kinase inhibitor 1C	*Cdkn1c*	5'-CTGGACAGGACAAGCGATCC-3'	5'-GCTGTTCTGCTGCGGAGGTA-3'
Cyclin dependent kinase inhibitor 2A	*Cdkn2a*	5'-GCTCTGGCTTTCGTGAACAT-3'	5'-CGAATCTGCACCGTAGTTGA-3'
B cell leukemia/lymphoma 2	*Bcl2*	5'- ATGCCTTTGTGGAACTATATGGC-3'	5'- GGTATGCACCCAGAGTGATGC-3'
BCL2-associated X protein	*Bax*	5'- TGAAGACAGGGGCCTTTTTG-3'	5'- AATTCGCCGGAGACACTCG-3'
BCL2-associated agonist of cell death	*Bad*	5'- AAGTCCGATCCCGGAATCC-3'	5'- GCTCACTCGGCTCAAACTCT-3'
Caspase 3	*Casp3*	5'-TGGTGATGAAGGGGTCATTTATG-3'	5'-TTCGGCTTTCCAGTCAGACTC-3'
Caspase 8	*Casp8*	5'- GTGAGCCGGCGTGGAACAGG-3'	5'- AGAGCTGTAACCTGTGGCCGAGT-3'
BCL2-related ovarian killer	*Bok*	5'- CTGCCCCTGGAGGACGCTTG-3'	5'- CCGTCACCACAGGCTCCGAC-3'
Catalase	*Cat*	5'-GCAGATACCTGTGAACTGTC-3'	5'-GTAGAATGTCCGCACCTGAG-3'
Glutathione peroxidase	*Gpx*	5'-TTCGGACACCAGGAGAATGG-3'	5'-TAAAGAGCGGGTGAGCCTTC-3'
Glutathione reductase	*Gsr*	5'- CAGTTGGCATGTCATCAAGCA-3'	5'-CGAATGTTGCATAGCCGTGG-3'
Peroxisome proliferator-activated receptor alpha	*Ppara*	5'-TGAACAAAGACGGGATG-3'	5'-TCAAACTTGGGTTCCATGAT-3'
Peroxisome proliferator-activated receptor gamma	*Pparg*	5'-TGTGAGACCAACAGCCTGACGG-3'	5'-GTCCTGAATATCAGTGGTTCACCGC-3'
Estrogen receptor 2	*Esr2*	5'-GGAATCTCTTCCCAGCAGCA-3'	5'-GGGACCACATTTTTGCACTT-3'

Table 5.1 (cont.)

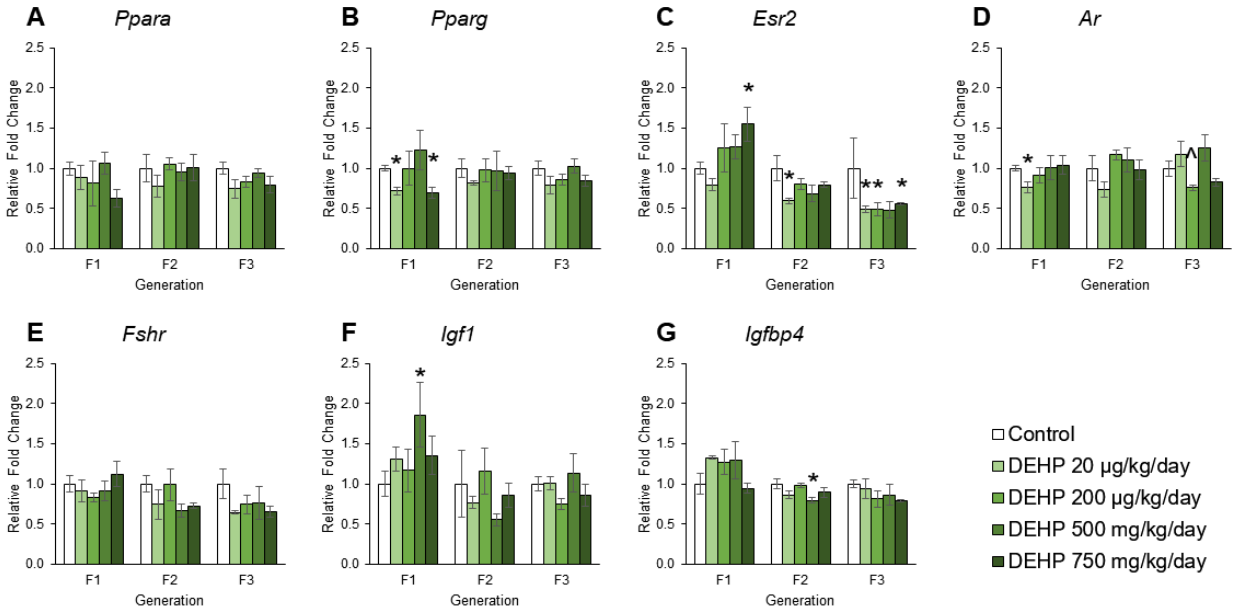
Gene name	Symbol	Forward primer	Reverse primer
Follicle-stimulating hormone receptor	*Fshr*	5'-AGCAAGTTTGGCTGTTATGAGG-3'	5'-GTTCTGGACTGAATGATTTAGAGG-3'
Insulin-like growth factor 1	*Igf1*	5'-ATCCCAAGCCCTGTTTGGTT-3'	5'-TGCCCCCAGTGTTTTGAAGT-3'
insulin-like growth factor binding protein 4	*Igfbp4*	5'-TTCTCAACTCAAGGCCCACG-3'	5'-GGGCGTTCAATGTTCACCAC-3'
DNA methyltransferase 1	*Dnmt1*	5'-GGAAGGCTACCTGGCTAAAGTCAAG-3'	5'-ACTGAAAGGGTGTCACTGTCCGAC-3'
DNA methyltransferase 3a	*Dnmt3a*	5'-TGGAGAATGGCTGCTGTGTGAC-3'	5'-CACTCATCCCGTTTCCGTTGG-3'
DNA methyltransferase 3b	*Dnmt3b*	5'-AGTGACCAGTCCTCAGACACGAAG-3'	5'-ATCAGAGCCATTCCCATCATCTAC-3'
Ten-eleven translocation enzyme 1	*Tet1*	5'-ACATCCCACAGACCGAAGAT-3'	5'-TTCTGGGGTTTTCACTCCTC-3'
Ten-eleven translocation enzyme 2	*Tet2*	5'-AGAGCCTCAAGGCAACCAAAA-3'	5'-ACATCCCTGAGAGCTCTTGC-3'
Ten-eleven translocation enzyme 3	*Tet3*	5'-CCGGATTGAGAAGGTCATCTAC-3'	5'-AAGATAACAATCACGGCGTTCT-3'

Figure 5.1 The Database of Annotation, Visualization, and Integrated Discovery functional analysis



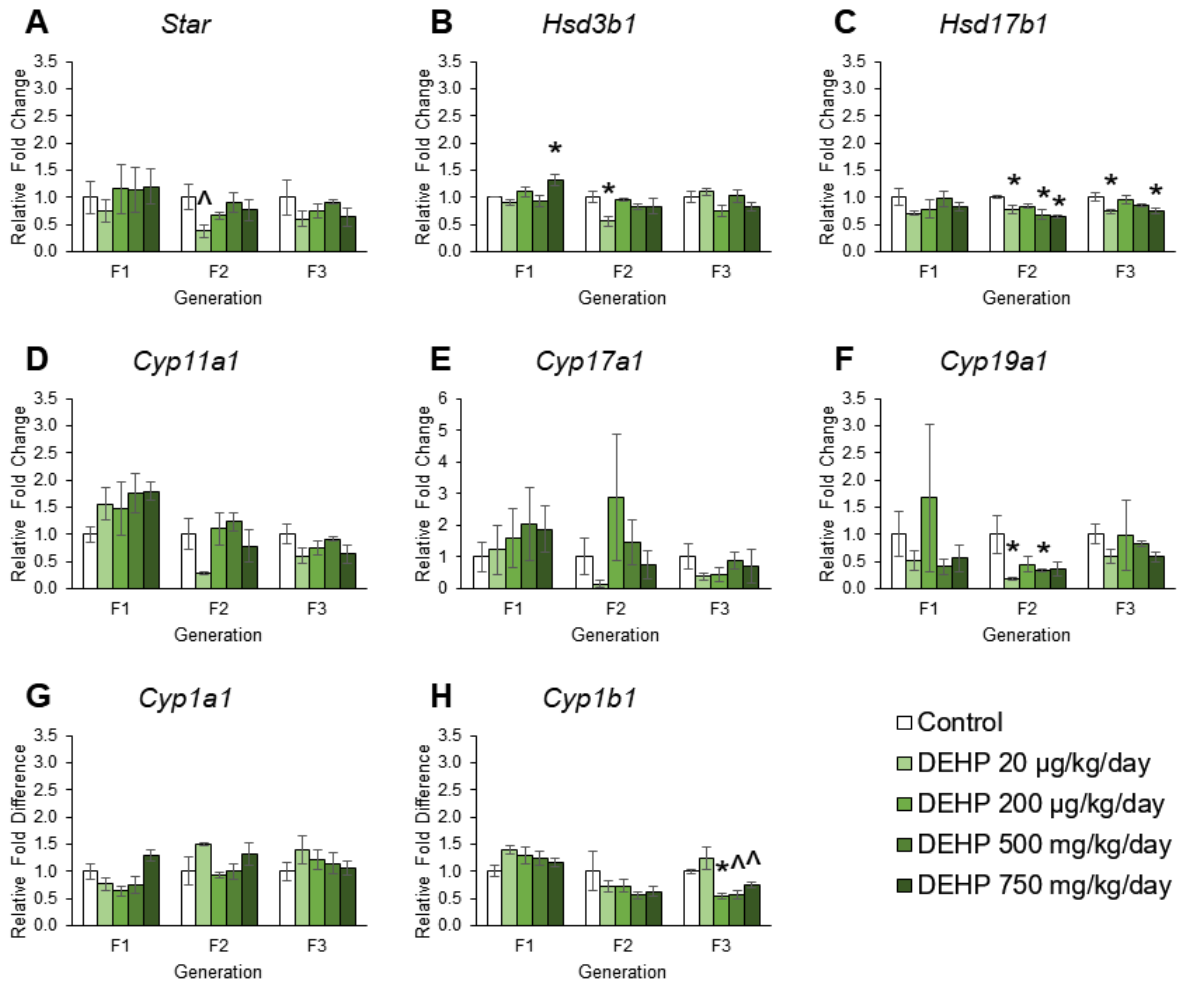
Data obtained from the RNA sequencing were functionally analyzed using The Database of Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics version 6.8. A total of 177 genes were entered into DAVID (false discovery rate < 0.62 and $p < 0.007$) for functional annotation analysis. “Gene_Ontology” results yield 5 annotation clusters (A) and “Pathways” results yield 1 annotation cluster (B) with a significant enrichment score ≥ 1.

Figure 5.2 The effects of DEHP exposure on steroid hormone receptors and insulin-like growth factors in the F1 – F3 generations



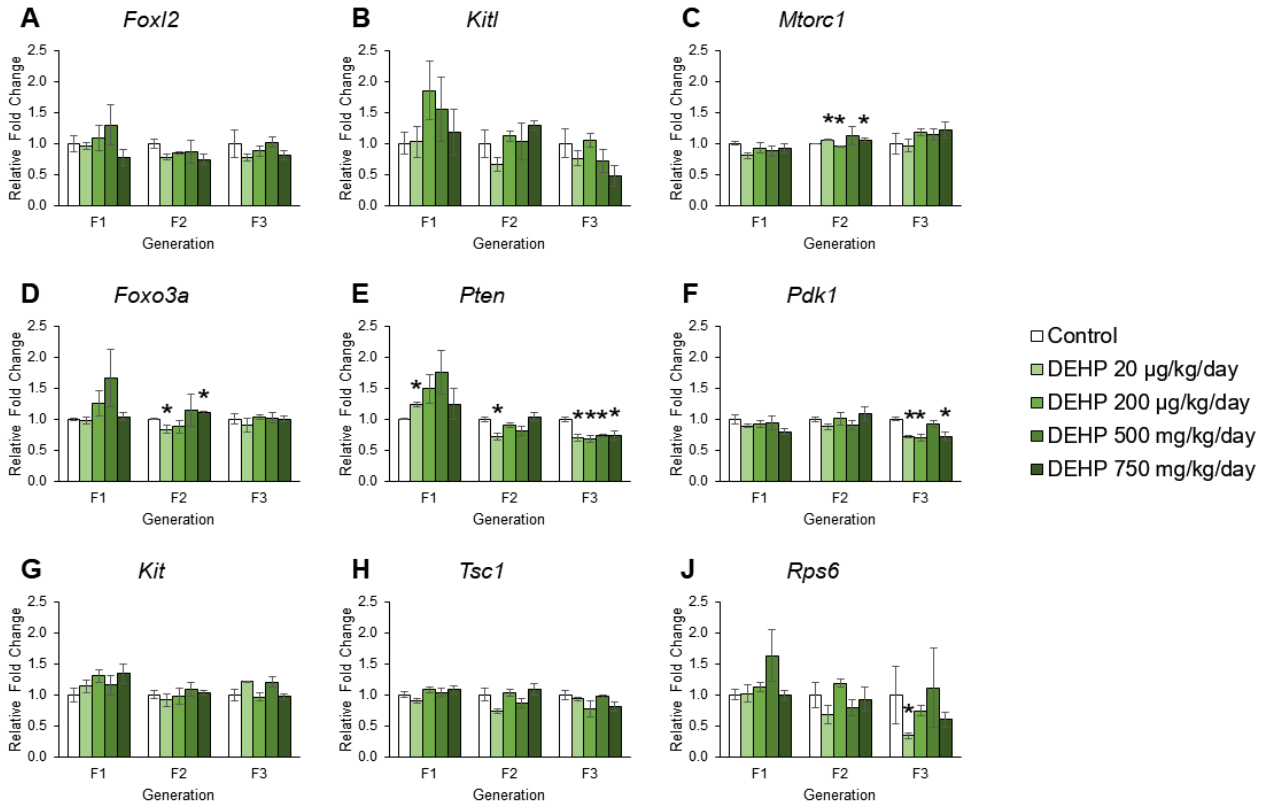
The effects of prenatal and ancestral DEHP exposure on steroid hormone receptors and insulin-like growth factors in PND 21 ovaries in the F1 – F3 generations. All gene expression is relative to the housekeeping gene, *Bactin*, and the relative fold change is normalized to 1 for control. Graphs represent mean ± SEM from 3 – 6 ovaries per treatment group. * $p \leq 0.05$ (significant difference compared to control with generation), $0.05 < \wedge p < 0.10$.

Figure 5.3 The effects of DEHP exposure on steroidogenesis and estradiol metabolism in the F1 – F3 generations



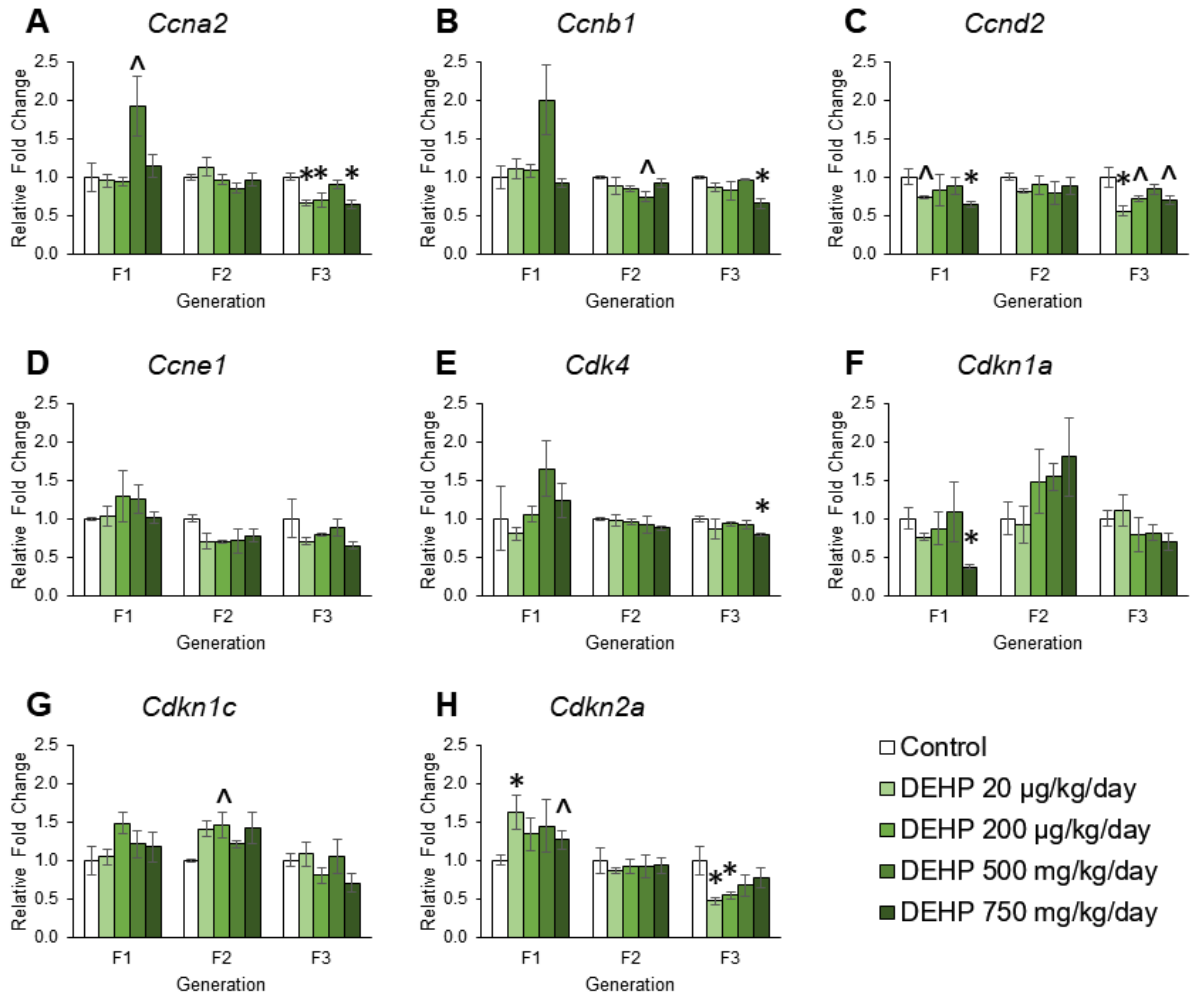
The effects of prenatal and ancestral DEHP exposure on steroidogenesis and estradiol metabolism in PND 21 ovaries in the F1 – F3 generations. All gene expression is relative to the housekeeping gene, *Bactin*, and the relative fold change is normalized to 1 for control. Graphs represent mean ± SEM from 3 – 6 ovaries per treatment group. * $p \leq 0.05$ (significant difference compared to control with generation), $0.05 < \text{^} < 0.10$.

Figure 5.4 The effects of DEHP exposure on the phosphoinositide 3-kinase pathway in the F1 – F3 generations



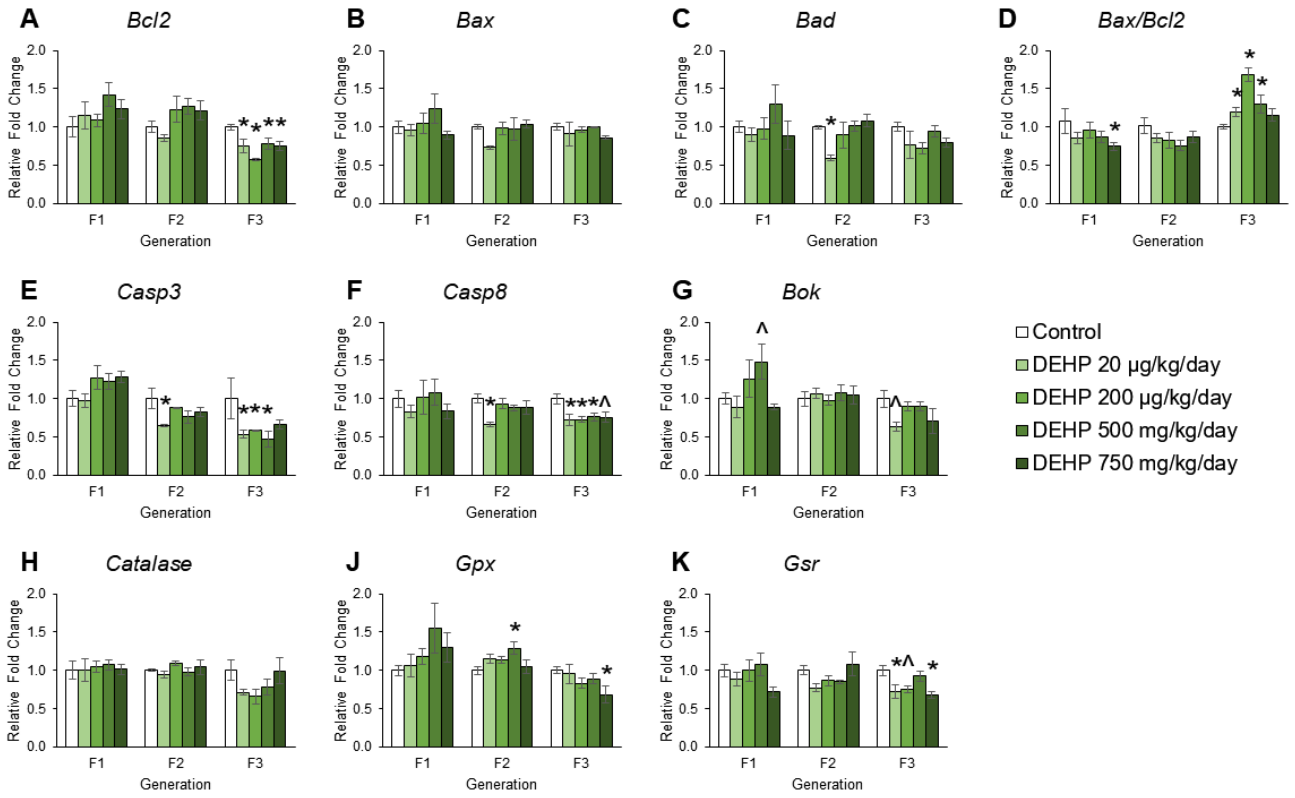
The effects of prenatal and ancestral DEHP exposure on the phosphoinositide 3-kinase pathway in PND 21 ovaries in the F1 – F3 generations. All gene expression is relative to the housekeeping gene, *Bactin,* and the relative fold change is normalized to 1 for control. Graphs represent mean ± SEM from 3 – 6 ovaries per treatment group. * $p \leq 0.05$ (significant difference compared to control with generation), $0.05 < \wedge p < 0.10$.

Figure 5.5 The effects of DEHP exposure on cell cycle regulators in the F1 – F3 generations



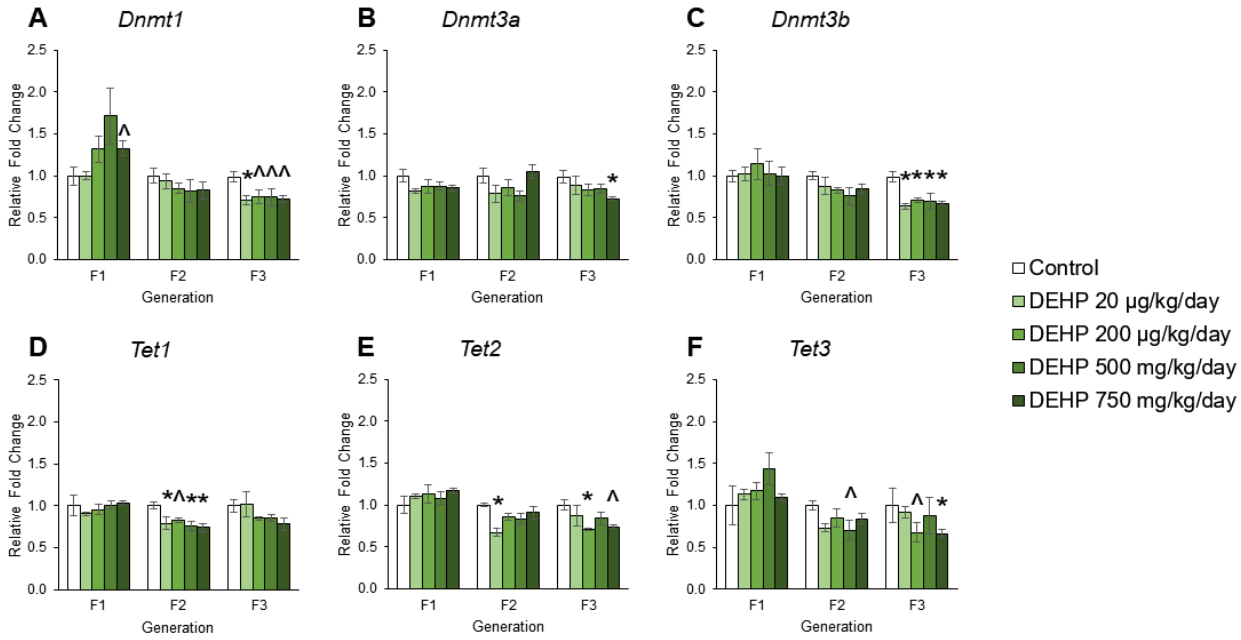
The effects of prenatal and ancestral DEHP exposure on cell cycle regulators in PND 21 ovaries in the F1 – F3 generations. All gene expression is relative to the housekeeping gene, *Bactin,* and the relative fold change is normalized to 1 for control. Graphs represent mean ± SEM from 3 – 6 ovaries per treatment group. * $p \leq 0.05$ (significant difference compared to control with generation), $0.05 < \text{^} p < 0.10$.

Figure 5.6 The effects of DEHP exposure on the B-cell lymphomas/leukemia-2 family and oxidative stress factors in the F1 – F3 generations



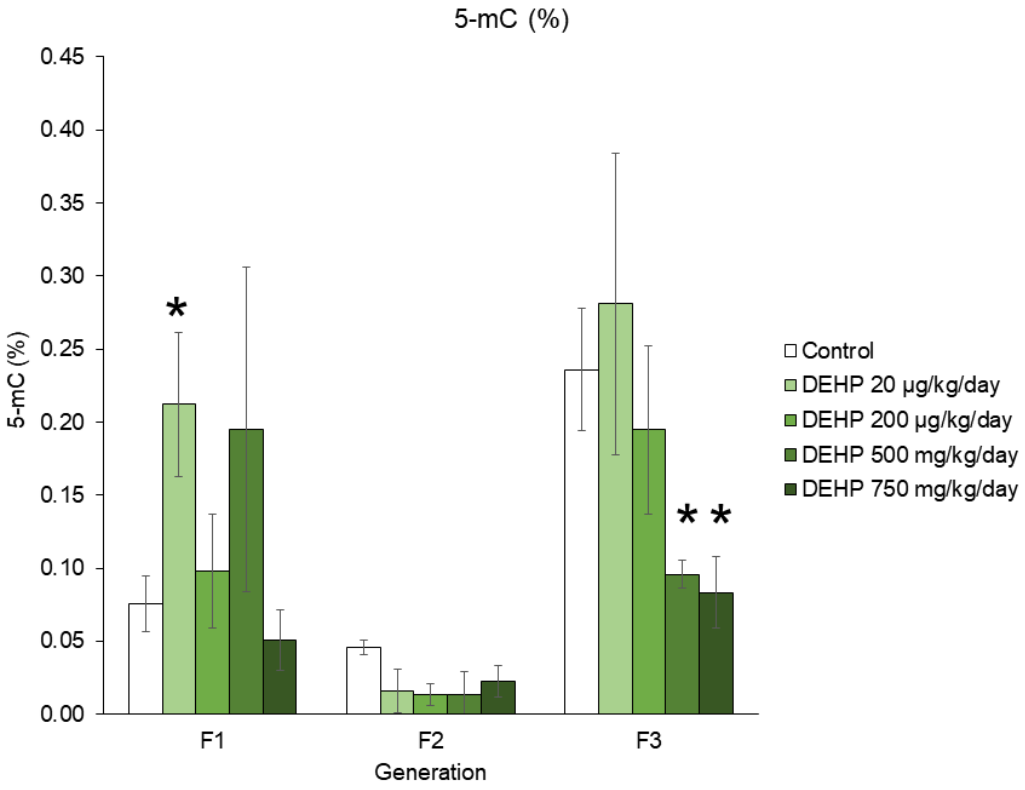
The effects of prenatal and ancestral DEHP exposure on the B-cell lymphomas/leukemia-2 family and oxidative stress factors in PND 21 ovaries in the F1 – F3 generations. All gene expression is relative to the housekeeping gene, *Bactin,* and the relative fold change is normalized to 1 for control. Graphs represent mean ± SEM from 3 – 6 ovaries per treatment group. * $p \leq 0.05$ (significant difference compared to control with generation), $0.05 < ^{} p < 0.10$.

Figure 5.7 The effects of DEHP exposure on DNA methyltransferases and ten-eleven translocation enzymes in the F1 – F3 generations



The effects of prenatal and ancestral DEHP exposure on DNA methyltransferases and ten-eleven translocation enzymes in PND 21 ovaries in the F1 – F3 generations. All gene expression is relative to the housekeeping gene, *Bactin*, and the relative fold change is normalized to 1 for control. Graphs represent mean ± SEM from 3 – 6 ovaries per treatment group. * $p \leq 0.05$ (significant difference compared to control with generation), $0.05 < \text{^} < 0.10$.

Figure 5.8 The effects of DEHP exposure on the percentage of 5-methyl cytosine in the F1 – F3 generations



The effects of prenatal and ancestral DEHP exposure on the percentage of 5-methyl cytosine in whole ovaries at PND 21 in the F1 – F3 generations. Graphs represent mean ± SEM from 3 – 7 ovaries per treatment group. * $p \leq 0.05$ (significant difference compared to control with generation).

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CHAPTER 6

Summary, Conclusions, and Future Directions

6.1 Summary, Conclusions, and Future Directions

My doctoral dissertation work was designed to determine the developmental and reproductive toxicity of DEHP exposure in female mice. DEHP is a widely used plasticizer in consumer products and is a known developmental and reproductive toxicant [1-3]. DEHP and its bioactive metabolites are found in human tissues such as amniotic fluid, cord blood, breast milk, and human follicular fluid [4]. The presence of DEHP in tissues vital for reproduction and development suggests that DEHP exposure occurs at various developmental stages; thus, posing as a potential toxicant for several generations. Many studies have been conducted on the toxic effects of DEHP on the female reproduction. However, most of these studies focused on one generation or focus on adult exposure [1, 2, 5, 6]. Understanding the effects of environmentally relevant DEHP exposure on female reproduction across generations is critical so that we can better understand the potential risks of DEHP on human and animal health. Collectively, my study shows that environmentally relevant levels of DEHP disrupts ovarian functions, impairs reproductive outcomes, and decreases expression of genes necessary for normal ovarian functions across generations in female mice.

In Chapter 3, I tested the hypothesis that prenatal DEHP exposure disrupts ovarian functions in a transgenerational manner. I found that prenatal and ancestral DEHP exposure disrupted folliculogenesis in a multigenerational manner and accelerated germ cell transition in a transgenerational manner. Specifically, in the F1 generation, prenatal DEHP exposure decreased folliculogenesis and decreased the percentage of atretic follicles. In the F2 generation, DEHP exposure disrupted folliculogenesis. In the F3 generation, ancestral DEHP exposure accelerated

transition of germ cells to primordial follicles in neonatal ovaries. The altered folliculogenesis in the ovary suggests that DEHP exposure directly targets maturing follicles and the changes in germ cell numbers suggest that DHEP targets ovarian development. Future studies should test the health of ovarian follicles by isolating and culturing them to monitor their growth and hormone production. Additionally, studies should test the health of the oocyte by performing *in vitro* fertilization on isolated follicles. Further, future studies should investigate the effects of folliculogenesis at older ages and determine if DEHP induces premature ovarian failure.

In Chapter 3, I measured serum hormone levels and found that prenatal DEHP exposure disrupted serum sex steroid hormone levels in female mice. In the F1 generation, prenatal DEHP exposure increased serum 17β-estradiol. In the F2 generation, DEHP exposure disrupted serum progesterone levels. The altered steroidogenesis suggests that DEHP exposure targets serum sex steroid hormone levels either through synthesis or metabolism. These hormones are critical for normal reproductive health and function and are also critical for bone, heart, and mental health [7-13]. Additional studies should determine whether DEHP exposure disrupts enzymes that metabolize sex steroid hormones in the liver. Enzymes that metabolize sex steroid hormones can have an impact on the serum sex steroid hormone level in the body [14]. In addition, studies should determine the impact of DEHP exposure on other sex steroid hormone synthesizing organs such as the adrenals, which secrete precursor hormones such as progesterone and androgens [15], so that we can better understand the impact of DEHP on steroidogenesis. Further, additional studies should measure serum steroid hormone levels at all phases of the estrous cycle to determine if DEHP exposure targets a particular phase.

In Chapter 4, I hypothesized that prenatal and ancestral exposure to DEHP disrupts female reproductive outcomes in both a multigenerational and transgenerational manner. I found

that prenatal and ancestral DEHP exposure accelerated the onset of puberty and estrous cyclicity. In the F1 generation, prenatal DEHP exposure accelerated the onset of puberty and disrupted post-pubertal cyclicity. In the F2 generation, DEHP exposure accelerated the onset of puberty and disrupted post-pubertal cyclicity and adult estrous cyclicity. In the F3 generation, ancestral DEHP exposure accelerated the onset of puberty and disrupted post-pubertal cyclicity and adult estrous cyclicity. Our data show that DEHP exposure disrupts pubertal onset in female mice. Pubertal onset is governed by a feedback loop between the hypothalamus, pituitary, and gonads [16]. Additional studies are needed to better understand the mechanism by which DEHP induces precocious puberty. Specifically, future studies should determine whether DEHP exposure disrupts GnRH firing and production of gonadotropin hormones around the time of puberty.

In Chapter 4, I found that prenatal DEHP exposure disrupts birth outcomes and decreases fertility-related indices in a multigenerational manner. In the F1 generation, prenatal DEHP exposure decreased the mating index and pregnancy index. In the F2 generation, DEHP exposure increased litter size and decreased the gestational index. My data show that DEHP exposure directly affects the fertility of female mice. Additional studies are necessary to determine the mechanism of action. Specifically, additional studies should investigate the effects of DEHP on mating behavior and determine if the decreased mating index is due to a behavioral change caused by DEHP. My study found that the majority of infertility issues caused by DEHP were due to mid-gestation infertility. Possible causes of mid-gestation infertility include disrupted uterine function, immune response, fetal development, or hormone signaling. Therefore, it is critical for future studies to examine the cause of breeding and pregnancy complications. Future studies that investigate the uterus are critical because the uterus is fundamental for the maintenance of a pregnancy.

In Chapter 5, I hypothesized that prenatal and ancestral DEHP disrupted gene expression of pathways critical for ovarian health and DNA methylation mechanisms. I found that prenatal and ancestral DEHP exposure disrupted gene expression of various pathways in the ovary in a multigenerational and transgenerational manner. In the F1 generation, prenatal DEHP exposure dysregulated the expression of genes in the cell cycle regulators and peroxisome-proliferator activated receptors in the whole ovary. In the F2 generation, DEHP exposure decreased the expression of steroidogenic enzymes, disrupted the expression of PI3K factors, and decreased the expression of pro-apoptotic factors in the whole ovary. In the F3 generation, ancestral DEHP exposure decreased the expression of PI3K factors, cell cycle regulators, apoptotic factors, oxidative stress factors, and estrogen receptor 2 in the whole ovary. Future studies should investigate the effects of DEHP exposure on protein expression in the ovary. Although my data show that DEHP exposure decreases gene expression in the ovary, it is critical to know if DEHP disrupts the expression of proteins.

In Chapter 5, I also found that prenatal and ancestral DEHP exposure dysregulated the expression of *Dnmt*s and *Tet*s and altered DNA methylation levels in both a multigenerational and transgenerational manner. In the F1 generation, prenatal DEHP exposure increased the expression of *Dnmt1* and increased the percentage of 5-mC in the whole ovary. In the F2 generation, DEHP exposure decreased the expression of *Tet*s. In the F3 generation, ancestral DEHP exposure decreased the expression of *Dnmt*s, *Tet*s, and decreased the percentage of 5-mC in the whole ovary. Future studies should investigate the effects of DEHP exposure on gene expression and DNA methylation in single cells. The ovary is a heterogeneous organ and it is critical to know which cells gene expression changes are occurring. It is especially important to know if DEHP induces DNA methylation changes in the germ cell. Additionally, future studies

should determine the protein expression of DNMT and TET in the ovary. Protein expression and activity of DNMT and TET are critical for the execution of DNA methylation [17-24]. Further studies should determine the methylation profile of promoter regions of genes that were differentially expressed due to DEHP exposure. Such studies would determine whether DNA methylation specifically targets gene expression changes. However, it is necessary that additional studies determine whether DEHP acts through additional epigenetic pathways.

Further studies should investigate the effects of prenatal and ancestral exposure to DEHP on the ovary in older mice. Although I observed significant changes in ovarian function and health and early reproductive outcomes in the female mice, it is critical to observe the life-long effects of DEHP exposure. Therefore, future studies should examine the health of the ovary in mice significantly older than postnatal day 60. Additionally, given that humans and animals are continuously exposed to DEHP on a daily basis, it is critical to have future studies that expose animals to DEHP on a daily basis to better understand the effects of DEHP on female reproductive health. Future analyses should also investigate different developmental exposure windows to DEHP to determine the mechanisms that causes transgenerational inheritance of disease and dysfunction. Although there are studies that expose mice to DEHP during gestation and the neonatal time period [25], it is important to determine the most sensitive window of exposure to induce transgenerational inheritance of disease in females.

Overall, my doctoral dissertation work indicates that prenatal and ancestral DEHP exposure disrupts female reproductive outcomes in a multigenerational and transgenerational manner. Specifically, DEHP exposure disrupts ovarian health and function by altering steroidogenesis in a multigenerational manner and folliculogenesis in a transgenerational manner. Moreover, DEHP exposure disrupts female reproductive outcomes in all generations of

female mice. Further, DEHP exposure dysregulates the expression of *Dnmt*s and *Tet*s and alters DNA methylation levels in both a multigenerational and transgenerational manner. These findings are important for the public health because humans and animals are continuously exposed to DEHP and our findings show that the effects of DEHP on female reproduction span generations. Further, the environmentally relevant doses of DEHP used in my doctoral dissertation work provide doses relevant to human exposure.

6.2 References

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